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(54) Title: HUMAN T CELL MONOCLONE, PROCESS FOR ITS PRODUCTION AND ITS USE, DIAGNOSTIC OF INFECTIOUS DISEASES, AUTOIMMUNE DISEASES, T-CELL MEDIATED ALLERGIES AND CANCER

(57) Abstract

The present invention concerns a human T cell monoclone which is highly proliferative and antigen mono-specific. The present invention is also related to the process for the production of the human T cell monoclone according to the invention and to the use of human T cell monoclone according to the invention for the treatment of infectious diseases, autoimmune diseases, T cell mediated allergies and cancer.

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HUMAN T CELL MONOCLONE, PROCESS FOR ITS PRODUCTION AND ITS USE, DIAGNOSTIC OF INFECTIOUS DISEASES, AUTOIMMUNE DISEASES, T-CELL MEDIATED ALLERGIES AND CANCER

Field of the Invention

This invention is in the field of immunology, immunotherapy and immunodiagnosis. More specifically, the invention is directed to highly proliferative and antigen-specific T-cell monoclones and a process for its production and its use for the treatment and diagnosis of infectious diseases, autoimmune diseases, T-cell mediated allergies and cancer.

Background of the invention and state of the art

The immune system protects the host against foreign intruders (antigens) or harmful agents. It is regulated in a sophisticated network, comprising among others T cells and B cells which are programmed to carry out specific tasks. An inappropriate functioning of the immune system can lead to an immune deficiency, as observed in AIDS, or to a non balanced immune regulation, as observed in autoimmune diseases. T cells play a major role in the control of the immune system. Many immune-related diseases known to-day are associated with a deficient or abnormal T cell function.

Recent advances in the methodology for establishing antigen-specific T cell lines in vitro have helped a great deal in our understanding of the molecular and cellular immunology. These advances have made it possible to analyze the T cell receptor genes and the mechanisms by which the T cell repertoire diversity is generated and they have provided valuable information on the role of immune-response gene products in antigen presentation. In particular, clonal analysis of antigen-specific T cells has provided an

opportunity to define the pathologic role of certain T cell populations in the pathogenesis of several human diseases. Ground rules for T cell recognition of proteins and synthetic peptides are now being established. On the basis of this information it is possible to design vaccines that will elicit either MHC Class I or Class II restricted T cell immunity. Moreover, in vitro generated antigen-specific T cell lines and clones can be of enormous practical help to identify disease mechanisms and to develop immunotherapeutic and immunodiagnostic strategies.

There are several groups of human diseases where antigen-specific T cell lines and clones could be of valuable help in designing an effective treatment. Autoimmune diseases, in particular, are a group of diseases sharing a common feature, that is, a deficit in the immune regulation of autoreactive T cells. (reviewed by Brostoff et al. eds. Clinical Immunology, Gower Medical Publishing, London-New-York, 1991). Examples of human autoimmune diseases include Graves' disease, multiple sclerosis (MS), rheumatoid arthritis (RA), Myasthenia gravis (MG), type I diabetes etc. In all known autoimmune diseases, autoreactive T cells or B cells producing autoantibodies are activated and clonally expanded to mount an attack on target tissues of the host. The process often leads to a recruitment of inflammatory cells, including macrophages, gamma delta T cells and T cells capable of producing inflammatory cytokines, followed by a destruction of the tissues involved (reviewed in Deodhar et al., Clin. Biochem. 25, 181, 1992).

Multiple sclerosis is a chronic inflammatory demyelinating disease of the central nervous system, characterized by infiltrations of T lymphocytes and macrophages into white matter of the brain and by locally produced inflammatory cytokines and antibodies in the central nervous system (Selmaj et al., Ann.

Neurol. 23, 339, 1988; Cross et al., J. Neuroimmunol. 33, 237, 1991). These processes are associated with demyelinations and neurological dysfunctions. Most often the infiltrating cells are autoimmune in nature and they act as effectors in a process whereby self proteins are recognized and myelin tissue is destroyed. Autoimmune mechanisms mediated by autoreactive T cells hold a central position in a cascade of events leading to demyelination (Reviewed in Zhang et al., Intern. Rev. Immunol. 9, 183, 1992).

Our knowledge of MS and other autoimmune diseases is largely guided by studies in experimental animals. Experimental Autoimmune Encephalomyelitis (EAE) is a paralytic disease of the central nervous system, which shares many similarities with MS. It therefore is generally regarded as an animal model for MS. EAE can be induced by activated T cells specific for MBP or Proteolipid Protein (PLP). In humans, potential pathologic effects of MBP-specific T cells can be illustrated in post-vaccination encephalomyelitis which develops in individuals who have received a rabies vaccine prepared from infected rabbit brain. Similar to experimental animals, MBP-specific T cells occur at an increased frequency in cerebrospinal fluid of patients with MS. These autoreactive T cells isolated from peripheral blood of patients are found in an activated state, suggesting their role in the disease process. Because of the active involvement of the immune system several current treatments of MS are based on non-specific immune suppression. However because of lack of specificity these treatments most often are associated with severe side-effects.

If MS is an inflammatory autoimmune disease mediated by autoreactive T cell responses to myelin antigens, it is theoretically feasible to design an immunotherapy to eliminate selectively these pathogenic

T cells. This speculation is largely based on studies in the EAE model.

The part of the T cells that distinguishes one T cell or group of T cells from another is the T cell receptor (TCR). Thus the TCR seems to be the most appropriate target for designing an effective and specific therapeutic strategy. An obvious requirement for the therapy is the specificity of the treatment for the particular TCR involved. This means that the population of pathogenic T cells must be homogeneous with regard to the TCR repertoire for recognizing the autoantigens involved. This condition seems to be met in EAE in Lewis rats and PL/J mice where encephalitogenic MBP-reactive T cells are restricted to limited epitopes on MBP and to a single TCR Vβ gene segment (Burns et al., J. Exp. Med. 169, 27, 1989; Acha-Orbea et al., Cell 54, 263, 1988). Various therapeutic strategies designed to target either at the TCR or the autoreactive T cells as a whole have shown to be effective in preventing the development of EAE in sensitized animals (Reviewed in Zhang et al., Intern. Rev. Immunol. 9, 183, 1992).

However unlike its animal counterparts, the situation in MS is more complex with respect to the epitope specificity and the TCR gene usage of MBP-specific T cells (Ben-Nun et al. Proc. Natl. Acad. Sci. 88, 2466, 1991). Furthermore the disease is more complicated by the involvement of Major Histocompatibility Complex (MHC) genes which are highly diverse and variable from one individual to another. The MHC gene products are important elements with which T cells are able to recognize an antigen. Thus, before a target structure commonly shared by these pathogenic T cells is defined, a specific immunotherapy would have to be tailored to a particular individual or a group of individuals sharing related MHC genes.

Activated MBP-specific T cells, when rendered non virulent, can prevent and treat EAE in experimental animals (Ben-Nun et al., Nature 292, 60, 1981). This procedure is termed T cell vaccination in analogy with microbial vaccinations to prevent infectious diseases. Both activation and attenuation are required for the vaccine to be effective in treating the disease. Attenuation can be achieved by either chemical modification or irradiation (Ben-Nun et al., Nature 292, 60, 1981). T cell vaccination has been found to be effective in preventing and treating several experimental autoimmune diseases. Whole live or attenuated T lymphocytes have been used as vaccines to treat or prevent, in addition to EAE, Experimental Autoimmune Thyroiditis (EAT), Adjuvant Arthritis (AA) and Experimental Autoimmune Uveitis (EAU) (Cohen, Immunol. Rev. 94, 5, 1986). Since the fine specificity of vaccination is dictated by the fine specificity of the T cell recognition, the TCR most likely is involved in the therapeutic or preventive effects. For example, two different MBP-specific T cell lines, each reactive to a different epitope of MBP, were found to vaccinate against EAE specifically induced by the particular epitope, indicating some form of anti-idiotypic immunity. However, when attempts were made to isolate clones of anti-idiotypic MBP-specific or thyroglobulin-specific T cells (in a thyroiditis model) from the uncloned cell lines, only clones producing disease, but not resistance, were obtained. This led to the hypothesis that appropriate aggregation or rigidification of cell membranes, by either hydrostatic pressure or chemical cross-linking, would yield cells which could induce protection more consistently. Similarly, low doses (sub-encephalitogenic) of MBP-specific cells were able to induce resistance to lethal EAE. The protective state was termed "counter-autoimmunity". This state involves T cell clones which

can specifically proliferate in response to the vaccinating T cells, can suppress effector clones in vitro (non-specifically, presumably through release of a suppressive lymphokine), and can adoptively transfer counter-autoimmunity in vivo. Such counter-autoimmunity is accompanied by suppressed delayed-type hypersensitivity (DTH) responses to the specific epitope and prevention or remission of clinical disease.

The biologic principles learned from the animal studies may be operating in MS as well, provided autoreactive T cells play a similar pathogenic role in MS. Thus, the immunotherapeutic strategies effective in the treatment of EAE may have provided some clues in designing specific treatments for MS.

Another group of diseases where antigen-specific T cells play an important role in the disease mechanisms are the T-cell mediated allergies such as the nickel-mediated allergy (Kapsenberg, M.L. et al. J. Invest. Dermatol. 98, 59-63, 1992). Eliminating these allergen-specific T cells could be of benefit to reduce the disease symptoms.

A further group of human diseases where antigen-specific T cell clones are currently applied for treatment are cancers. Although the pathogenesis of cancer remains unknown, it is generally accepted that a deficit in the host immunity against cancerous cells plays an important role in the development of malignant tumors (Miescher et al., J. Immunol. 136, 1899, 1986). Although the exact mechanisms why tumor-specific T cells fail to eliminate tumor cells are unknown, current efforts are being made to develop an immunotherapy aimed at an increase of the functional capacities of tumor-specific T cells. In this regard, tumor infiltrating lymphocytes (TIL) harbored at tumor sites attract most interest since they may represent an attempt of the tumor-bearing host to develop an immune

attack against the tumor (Vose et al., Semin. Haematol. 22, 27, 1985). TIL are comprised of heterogeneous populations of effector and immunoregulatory lymphocytes and monocytes (Whiteside et al., Int. J. Cancer. 37, 803, 1986). Recent evidence obtained from animal studies has shown that adoptive transfer of TIL into the tumor-bearing host is able to mediate significant anti-tumor effects or even to induce total tumor regression (Rosenberg et al., Science 223, 1318, 1986). Similarly in humans, tumor-specific T cells can be derived from TIL preparations and are found to lyse tumor targets in an antigen specific fashion. Typically, TIL are isolated from surgical tumor specimens and expanded to 10^9 - 10^{11} cells for adoptive transfer into a tumor-bearing recipient. Its potential application as an adoptive immunotherapy is currently being evaluated in pre-clinical studies and clinical trials (Rosenberg et al.,New.Engl. J.Med.319, 1676, 1988).

The T-cells that attack foreign antigens, autoantigens and tumors are immunologically important because these T-cells recognize specifically foreign antigens, autoantigens and tumors as their targets, and these specificities can be utilized for therapeutics and diagnostics of the diseases associated with a deficient or badly regulated function of foreign antigen specific T-cells, autoantigen specific T-cells and tumor specific T-cells.

Difficulties in T cell cloning have been a constant challenge to all immunologists to study T cell interactions at the clonal level. Cloning of human T cells by specific antigens has been generally used as the conventional approach. In this technique T cells are stimulated by antigenic peptides processed and presented by antigen-presenting cells. However, it has been a general experience that this T cell activation pathway does not necessarily stimulate every

single antigen-specific T cell. There are several lines of evidence suggesting that an inappropriate antigen presentation occurred during the interaction between an antigenic peptide and the T cell receptor induces clonal anergy which renders T cells unresponsive to the antigen (LaSalle et al., J. Exp. Med. 176, 177, 1992). In addition, a culture condition for T cell activation is difficult to optimize since it varies with the individual antigen involved and the functional characteristics of the individual clones. Thus, cloning of human T cells by antigen stimulation is largely hampered by a lower cloning efficiency and, therefore requires higher numbers of T cells for the cloning process. This gives rise to contamination of the clone preparation by other irrelevant cells and to poor growth characteristics.

This is illustrated by our attempt to clone MBP-specific T cells from the blood of human subjects by stimulation with MBP. Although these clones demonstrated clonal responses to a single antigenic peptide and expressed a single phenotype, PCR analysis revealed multiple Vβ gene products, indicating the oligoclonal nature of the clone preparation (Ben-Nun et al., Proceedings of National Academy of Science, 1991). These MBP-specific T cell "clones" usually maintain reactivity to MBP for a short period of time (usually two or three weeks) and subsequently deteriorate in culture. It is likely that repeated stimulation with the antigen induces unresponsiveness of the "clones" by down regulation (LaSalle et al., Journal of Experimental Medicine, 1992).

Until recently the clonality of the resultant clones was not appropriately analyzed. The term "T cell clone" is ambiguously based upon the completion of a cloning process, usually with a lower cloning efficiency, and the antigen reactivity of the "clone" preparation. Thus in the past, the lack of proof for a

unique genetic marker of the clone has added to the suspicion on the true clonality of these "clones" as described in many earlier publications, even though this may not directly affect their experimental outcome. Due to the advances and the implementation of molecular biotechnology, in particular the PCR techniques, in T cell cloning procedures it has been possible to provide more evidence for the monoclonality of a particular cell preparation.

Summary of the invention

The present invention relates to a population of human T cell monoclones which is highly proliferative in the presence of an antigen to which human T cells forming this population are specific. The population of human T cell monoclones is characterized by its full biological purity in that it remains free of contaminating cells at all stages of subsequent culture development.

Preferably, the human T cell monoclone population of the present invention is characterized in that it gives rise to a single TCR V gene expression. It is also characterized in that it possesses a unique TCR V-D-J DNA sequence. Also preferred are T cell monoclone populations comprising cells of either the CD4 or the CD8 phenotypes.

The antigen for which the human T cells of the population of human T cell monoclones of the invention are specific and in the presence of which the population is proliferative is preferably a tumor cell or an immunogenic portion thereof, an auto-antigen. Preferred other antigens include Myelin antigens or immunogenic portions thereof, particularly the Myelin basic protein, the proteolipid protein, the Myelin-associated-glycoprotein, the Myelin-Oligodendrocyte-Glycoprotein and/or mixtures thereof, more particularly

an epitope of the 84-102 region or the 149-170 region of the amino acid sequence of Myelin Basic Protein.

The antigen to which the human T cells forming the population of human T cell monoclones are specific can also be a foreign antigen such as a Tetanus Toxoid antigen or an allergen that is mediating the allergy through T cells.

Also within the scope of the present invention is a method for the production of a population of human T cell monoclones which is highly proliferative in the presence of the antigen to which the human T cells are specific and/or any other T cell stimulating agent, and which is characterized by its full biological purity in that it remains free of contaminating cells at all stages of subsequent culture developement. The method comprises:

1) providing a human T cell line responsive to the antigen;

2) single cell cloning the T cell line and stimulating the resulting T cell clone with a T cell stimulating agent in the presence of autologous or allogeneic feeder cells to produce populations of human T cell monoclones; and

3) selecting the monoclone population having the desired TCR-specific characteristics.

Preferably, the human T cell line used in the method of the present invention is taken from peripheral blood lymphocytes. As for the T cell stimulating agent, it is preferably selected from the group consisting of lectines, preferably PHA and/or ConA, lymphokines, preferably Interleuken-2(IL-2) and/or a recombinant IL-2 (r-IL2) mitogenic antibodies against CD3 and other cell surface molecules and/or a mixture thereof.

Also within the scope of the present invention is a homogeneous population of T cell receptors from human T cell monoclones forming the

population of the present invention or the antigen-specific portion thereof and/or a mixture of selected populations or portions.

Also within the scope of the present invention is a therapeutic agent for the treatment of autoimmune diseases, T-cell mediated allergies, infections and cancer. The therapeutic agent comprises an effective amount of a population or a mixture of selected populations of T cell monoclones according to the invention.

The invention also relates to a vaccine composition for conferring upon humans active immunity against other immune diseases. The vaccine composition comprises an effective amount of a homogenous population of T cell receptors from human T cell monoclones according to the invention or a mixture of selected populations of T cell receptors obtained from the population of human T cell monoclones according to the invention or an antigen-specific portion thereof.

Also within the scope of the invention is a method for the treatment of a patient suffering from a condition caused by one or more antigens associated with this condition and obtainable from a biological sample of the patent. The method comprises vaccinating the patent with an adoptively transferring to the patient an amount of a human T cell monoclone population sufficient to generate the appropriate immune response to at least partially alleviate the condition. The human T cell monoclone population is responsive to the antigen and has a full biological purity in that it remains free of contaminating cells at all steps of culture development.

The invention also relates to a kit for preparing a population of human T cell monoclones which is highly proliferative in the presence of the antigen of the type which may be held responsible of a particularly diagnosed disease and for the subsequent

preparation of a population of the identified T cell monoclones. The kit comprises:

1) an essential antigen and its peptide specific to the diagnosed disease in sufficient amounts to generate cell lines responsive to the antigen from a biological sample;

2) means for plating the human T cell lines at very low cell densities;

3) a T cell stimulating agent for growing the human T cells at low density; and optionally

4) protocols and essential reagents for the characterization of the T cell monoclones.

The invention also relates to a diagnostic kit which comprises an appropriate solid support for immobilizing a biological sample containing a specific T cell responsive to an antigen associated with the condition to be diagnosed, means for at least immobilizing the specific T cell on the support and an antibody to a T cell monoclone receptor specific for the antigen associated with the condition to be diagnosed.

The population of human T cell monoclones of the present invention is particularly useful to maintain T cell monoclones in a long term culture in order to reach a sufficient amount of cells to prepare appropriate therapeutic agents. There is a clear need for a diagnostic and a therapeutic agent capable in a specific manner, of detecting, preventing, suppressing and/or treating infectious and immune-related diseases and/or cancer.

The human T cell monoclone population of the present invention can be expanded to a sufficiently high amount to be used either in a diagnostic kit or as a therapeutic agent, such as a vaccine or a preparation of T cells to be used for adoptive immune therapy. The human T cell monoclone population can also be used as a therapeutic agent to prevent, suppress and/or treat

infectious and immune-related diseases and/or cancer, without causing generalized suppression of immunity as it is the case with most current immuno-therapeutic and immuno-pharmacological approaches of the state of the art.

The present invention also provides a method for the in vitro preparation of foreign antigen specific, autoantigen specific and tumor specific T cell monoclone populations which minimizes or even eliminates the problems of contaminating cells associated with current methods of the state of the art.

SHORT DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the reactivity pattern of a panel of MBP-specific T cell lines to three MBP fragments.

Figure 2 represents the PCR analysis of TCR Vβ gene usage of MBP-specific T cell clones cloned by repeated MBP stimulation (panel A-B) and by PHA stimulation (panel C-D). Panel E represents single Vβ gene usage of a typical TSL clone cloned by the method of the invention.

Figure 3 illustrates a comparison of the cloning efficiencies of MBP-specific T cell lines by PHA and MBP stimulation.

Figure 4 represents the proliferative responses to the inoculates and control T cells and the changes in the frequency of MBP-specific T cells before and after each inoculation.

Figure 5 represents the relationship of changes in the frequencies of T cells reactive to MBP, TT and inoculates in recipients (GE and CW) and non-recipients (AH and GC).

Figure 6 represents the functional properties of the anti-clonotypic T cell lines.

Figure 7 represents the proliferative response of TSL lines to autologous and allogeneic tumor targets.

Figure 8 represents the cytotoxic activity of the TSL lines against autologous and allogeneic tumor cells, NK-sensitive K562 cell line, and NK-resistant Daudi cell line. The MCF7 cell line is a human breast cancer line.

Figure 9 represent the anti-clonotypic T cell responses to vaccine clones and changes in the estimated frequency of circulating MBP-reactive T cells in six patents with MS, before and after each inoculation.

Figure 10 is a schematic representation of the TCR gene organization and the specific sites within the target TCR potentially eliciting the anti-clonotypic T cell responses.

Detailed description of the invention

The invention relates to antigen-specific T-cell monoclonal populations and their use in diagnosis and treatment of various diseases such as infectious diseases, autoimmune diseases, T-cell mediated allergies and cancer.

Clonotypic regulation is one of the important components of peripheral regularotry mechanisms that keep autoreactive T cells in check. This regulartory network can be boosted by T cell vaccination to therapeutically deplete autoaggressive T cells in autoimmune pathologies, resembling traditional vaccination using attenuated autoreactive T cells as vaccines. For example, cellular and molecular interactions potentially involved in the clonotypic regulatory network result in therapeutic applications of T cell vaccination in human autoimmune diseases, such as multiple sclerosis.

Autoreactive T cells recognizing a variety of self-antigens represent part of the normal T cell repertoire and naturally circulate in the periphery. Common in many organ-specific autoimmune diseases, these autoreactive T cells undergo activation and clonal expansion, which represents the hallmark of the pathologic properties of autoaggressive T cells in the induction of autoimmune diseases. Activation of autoaggressive T cells renders them to acquire a different functional state and a homing pattern into the affected organ. Clonal expansion features not only an increase of autoaggressive T cells in their numbers but also a shift of their normally heterogeneous T cell receptor (TCR) repertoire towards an eliciting pathogenic epitope(s). The critical transaction from autoreactivity, a normal physiological state, to autoimmune pathology relates to the interplay between activation and clonal expansion of autoreactive T cells and an improper functioning of regulatory networks that keep them in control. One of the regulatory mechanisms involves the clonotypic network that regulates autoreactive T cells by interacting with their TCR clonotypic determinants. TCR hypervariable epitopes constitute clonotypic markers characteristic for an individual autoreactive T cell clone and recognizable by its regulators. The clonotypic interaction represents the "fine-tuning" of the regulatory network without affecting the remainder of the T cell repertoire. Recent investigations further suggest that such a clonotypic network is naturally operative in vivo and can be up-regulated in a clinical setting to therapeutically deplete autoaggressive T cells.

As pathogenic autoreactive T cells are viewed as pathogens in T cell-mediated autoimmune diseases, they can be used, when rendered avirulent by irradiation or pressure and chemical treatment, as vaccines to prevent and treat the diseases. The

principle of T cell vaccination is similar to traditional microbial vaccination against infectious agents. There is evidence that administration of attenuated autoreactive T cells as vaccines induces the regulatory networks to specifically suppress the cliciting autoreactive T cells (Ref. 7). T cell vaccination is effective in preventing and treating many experimental autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveitis, experimental diabetes model and adjuvant arthritis. The protective effect is long-lasting and specific since the autoreactive T cells used for vaccination only protected against the disease that they are able to induce.

The mechanism underlying T cell vaccination is not completely understood, but is thought to involve clonotypic network regulation directed at clonotypic determinants of a target TCR. Evidence supporting this notion comes from several observations.(1) $CD4^+$ and $CD8^+$ anti-clonotypic regulatory T cells are induced by and specifically recognize the immunizing clones. (2) These anti-clonotypic T cells are the major cellular component of the protective mechanism and are capable of conferring a specific protection to naive rats by adoptive transfer. Other regulatory T cells may also contribute to the protection by interacting with cellular markers other than the TCR clonotypes, such as the regulatory T cells identified as anti-ergotypic T cells that respond not to the TCR but to a marker associated with their state of activation.

Variable TCR region(s) involved in triggering the anti-clonotypic T cell responses in vivo are most likely to reside in hypervariable regions, such as complementary determining region-3 (CDR3) or less variable CDR2 regions.

T Cell vaccination offers a unique in vivo setting in which the clonotypic network selects a

relevant target epitope(s) to naturally regulate autoreactive T cells. In figure 10, there is provided a schematic representation of the TCR gene organization and the specific sites within the target TCR potentially eliciting the anti-clonotypic T cell responses in vivo.

Regognition of anti-clonotypic T cells to the junctional (CDR3) regions of the target TCR elicits a specific depletion of the immunizing T cell clone in the context of MHC class I molecules. Anti-clonotypic T cells with the other recognition pattern to a "cross-reactive" clonotype probably in CDR2 sequences of the variable regions react with, in addition to the immunizing clone, other T cells sharing the same clonotype.

Structural and functional features of autoreactive T cells in MS and their relevance in development of therapeutic strategies

In EAE, activated encephalitogenic T cells are the direct cause of the disease. Their TCR repertoire towards Myelin Basic Protein (MBP), a causal autoantigen, is rather limited with respect of both a limited epitope recognition and the V gene usage. Hence, the limited TCR repertoire provides suitable molecular targets for specific therapeutic interventions. Various TCR-based strategies have been developed to target at Vβ gene products or other attacking points within the TCR characteristic for the encephalitogenic T cells.

However, unlike the autoimmune disease model, the complexities of human autoimmune pathologies are often reflected by an obscure identity of eliciting autoantigens and a rather heterogeneous TCR repertoire of the autoreactive T cells involved. In MS, for example, T cell responses to myelin antigens, such as MBP, are implicated in the induction of autoimmune

pathology. In contrast to their rodent counterparts, human MBP-reactive T cells display a heterogeneous pattern of TCR Vβ genes. These T cells isolated from different MS patients use a broad spectrum of Vβ genes in response to MBP, even though the responses are relatively limited to the two immunodominant regions of MBP, residue 84-102 and residue 143-168. It is of interest to note that the Vβ gene usage pattern varies largely among MS patients but it appears to be restricted in a given individual. Studies conducted in the context of the present invention have revealed that the limited Vβ gene usage in a given MS individual rather represents a clonal expansion of MBP-specific T cells, as evidenced by their sharing of unique V-D-J and V-J juntional DNA sequence patterns. An example is given in Table 4 which illustrates a limited clonal origin(s) of MBP-reactive T cells isolated from a patient with MS. Based on these lines of evidence, it is reasonable to propose that MBP-specific T cells in MS undergo activation and clonal expansion that is marked by a skewed TCR repertoire towards a related epitope(s) and certain V gene usage. Consistent with this notion is our recent finding that in vivo activated MBP-reactive T cells often share an identical V-D-J sequence pattern with the clonally expanded population(s) in a given MS patient.

The heterogeneous expression of TCR V gene products among a general MS population would considerably perplex current attempts to develop TCR V gene-based therapeutic strategies. A treatment agent (e.g. a monoclonal antibody on a TCR peptide) designed to target at certain TCR V gene product(s) may be useful in one patient but is not suited for another, which hampers significantly its clinical usefulness. On the other hand, as clonal expansion of very limited MBP-specific T cell populations is a rather profound feature in MS, their restricted TCR repertoire provides

a uniform target structure in a given patient even though it varies between individuals. These clonally expanded T cells often account for more than 60%-80% of all MBP-specific T cells in a given individual with MS. Thus, a potential therapy may take the advantage of a predominant marker representative of clonally expanded MBP-reactive T cells but its applicability may be limited to given individuals, reflecting a dilemma in designing a suitable TCR-based therapeutic strategy.

From a therapeutic standpoint, there are two principal ways to halt T cell-mediated autoimmune processes either by blocking and depleting the pathogenic T cells or by boosting pre-existing regulatory mechanisms of the host. A potential drawback inherent in the former approach is that it requires frequent administrations of the remedy and its therapeutic effect is often short-term and diminishes with the withdrawal of the remedy. Thus, there is a need for an active therapy that mobilizes and up-regulates natural regulartory networks of the host to specifically restrain autoaggressive T cells. T cell vaccination appears to merit its place in the development of such a therapeutic strategy for various human pathologies. Furthermore, as a clonally expanded T cell population has a pathologic relevance in autoimmune disease, these T cells bearing a uniform clonotypic marker have an obvious therapeutic potential for immune intervention.

Method for the preparation of a human T cell monoclone population of the invention

A/ Generation of antigen-specific T cell lines

Generally, the T cells used in the production of the monoclone populations of the invention are selected according to the condition to be treated. For example, in the case of autoimmune diseases, patient

peripheral blood lymphocytes are used to derive the appropriate T cell lines. In the case of tumor specific lymphocytes, the cells are obtained from tumors excised from patients.

In other situations, it is also possible to develop monoclone populations from T cells found in the anatomic region associated with the condition. Examples of such situations include rheumatoid arthritis for which cells associated with this condition are found in the synovial fluid of the joints, and multiple sclerosis for which associated cells are found in the cerebro-spinal fluid.

In the case of foreign antigen specific T cell monoclone populations, specific cell lines can be generated from peripheral blood lymphocytes. As it will be seen from the examples which follow this description, this procedure was successfully applied to generate T cell lines specific to a Tetanus Toxoid antigen.

Various techniques for human T cell expansion have been described in the prior art. For example, one may refer to Zamvil et al, Nature 319: 355-358, (1985) and Nature 324: 258-260 (1986), Londei et al, Science 228-85-89 (1985), Londei et al Acta Endocrinol. 115 (suppl. 281): 86-89 (1987), Stamenkovic et al, Proc. Natl. Acad. Sci. USA 85: 1179-1183 (1988), Lipoldova et al, J. Autoimmun. 2: 1-13 (1989). However, it will be appreciated that the person skilled in the art can use the techniques referred to above or other methods to generate the appropriate antigen-specific cell lines. Furthermore, the person skilled in the art may also refer to the examples of the present application which provide specific procedures for generating MPB-specific T cell lines from peripheral blood, tumor specific lymphocytes from tumors excised from patients and the generation of Tetanus Toxoid specific T cell lines from peripheral blood.

In situations where the T cell lines are isolated from peripheral blood, peripheral blood lymphocytes are isolated and cultured in the presence of the antigen for a period of time ranging from 5 to 10 days. This time may vary depending on the number of reactive cells in the sample, the activation state of the cells, and the potency of the stimulating preparation. All these factors can be adjusted by the person skilled in the art.

The resulting cultures are restimulated with autologous antigen-presenting cells previously irradiated to prevent their proliferation and the antigen. The restimulation time may vary but will usually range between 5 and 12 days. In the case of tumor specific lymphocytes, it can, in some instances, be necessary to use surface-oxydized allogeneic cells to stimulate the lymphocytes periodically in the presence of an appropriate T cell stimulating agent.

The viable T-cell lines are then isolated and restimulated with the autologous antigen-presenting cells in the presence of the appropriate antigen for a period of time ranging from 5 to 12 days. The cell lines are then examined for their specific proliferation in response to the antigen in a proliferation assay.

B/ Single cell cloning of antigen-specific T cells

It is usually difficult to clone out true antigen-specific T cell clones because of problems usually associated with the autologous antigen-presenting cells, low cloning efficiencies and the induction of T cell tolerance during the antigen stimulation process. As a result of these problems, the general approach used to clone T-cells was to use between 10 and 30 cells per well. As a result, the

clone preparations are contaminated with unwanted T cells.

In the method of the present invention, the antigen-specific T cells are plated out at very low cell densities in the presence of irradiated autologous or allogeneic antigen-presenting cells and a potent T cell stimulating agent such as PHA and/or ConA, mitogenic antibodies against CD3 and other surface molecules and/or a mixture thereof. The person skilled in the art will appreciate that the T cell stimulating agents set forth above are provided as examples and that other T cell stimulating agents can also be used. During growth, cultures can be refed with fresh culture medium containing lymphocyte growth factors such as IL-2 that can be further expanded by alternate stimulation with the antigen and the T cell stimulating agents referred to above. It is to be noted that, apart from its growth factor propertie, IL-2 can also be used as a T-cell stimulating agent.

The single cell cloning approach is an important aspect of the present invention. Hence, it provides for higher cloning efficiencies, improved growth characteristics which allow for a large scale expansion of the clones, maintenance of antigen specificity after repeated expansions and monoclonality. In fact, with the method of the present invention, it is possible to grow a homogeneous population of several million cells in a period of time of 4 to 6 weeks. The use of a potent T cell stimulating agent avoids the contamination problems encountered with antigen-presenting cells.

Characteristics of the T cell population of the invention

As mentioned previously, the method of the present invention allows the production of homogeneous T cell monoclone populations that can be grown in

sufficient amounts to be used in therapy. Of course, the populations of the present invention are not restricted to cells recognizing a single immunogenic epitope or antigen. It is possible to develop cell line populations comprising mixtures of different clones that recognize different epitopes on one antigen. In such situations, it might be necessary at the beginning to conduct parallel single cell clonings in order to initially grow homogeneous populations that recognize a single epitope which can then be combined to generate the appropriate mixture.

A highly proliferative T cell clone can be defined by its stimulation index of at least 10 (CPM in the presence of the antigen/CPM in medium only), which is measured in a standard 3H-Thymidine uptake assay. Antigen-specific T cells are often tolerized after repeated antigenic challenge or by inappropriate antigen presentation. In this regard the invention offers a practical alternative by alternate stimulation of the clones with the antigen(s) and a non-specific stimulating agent such as PHA. This procedure ensures the specificity and responsiveness of a clone maintained in a long term culture.

The human T cell populations developed using the method of the present invention can therefore maintain a high degree of biological purity by remaining free of contaminating cells after numerous subculturing stages. This biological purity is explained in part by the absence of other cells having the ability to grow in the presence of the antigen to which the desired human T cells are specific. In therapeutic applications, it is important to maintain uniform characteristics in the cells forming the populations in order to ensure constant treatment efficacy.

Kit for the identification of human T cell monoclones and for the preparation of populations of human T cell monoclones

The kit can be used for the identification of those human T cell monoclones which are highly proliferative to an antigen of the type which may be held responsible of a particularly diagnosed disease and for the subsequent preparation of a population of the identified human T cell monoclones. Provided that the equipment required for the preparation of T cell vaccines is available on site, clinicians using the kit of the present invention are able to identify, from a biological sample of a patient, specific T cells responsive to a targeted antigen associated with the condition to be treated and to isolate and proliferate these specific T cells in sufficient amounts to use them for vaccination and treatment purposes.

Generally, the kit comprises the antigen, or an immunodominant peptide thereof, required to identify the specific T cell from the biological sample, means for plating the identified human T cell line at very low cell density and a T cell stimulating agent for growing the low density plated specific human T cell. Optional elements that can form part of the kit include reagents to evaluate the proliferation of the specific T cells prior to plating. The choice of these reagents is within the knowledge of the person skilled in the art.

Antigens comprised in the kit preferably include those antigens which are common to most patients suffering from the condition to be treated. It can be the whole molecule or peptides or fragments thereof containing the relevant immunodominant epitopes. Examples of such antigens include:

1) for rheumatoid arthritis:
   a) Collagen type II (1990 Rheumatol. Int. 10, 21-29

b) Heat Shock Proteins (1991 Int. Immunol. 3, 965-972)

c) Superantigens (1991 Proc. Natl. Acad. Sci. 88, 10921-10925)

2) for multiple sclerosis:

a) Myelin Basic Protein and immunodominant epitopes thereof (1992 Ann. Neurol. 32, 330-338 and 1990 Nature 346, 183-187)

b) Proteolipid Protein (1994 J. Exp. Med. Vol. 179)

3) for diabetes mellitus type I:

Glutamic acid decarboxylase (1993 J. Exp. Med. 177, 535-540)

4) for allergies:

different types of allergens mediated by lymphocytes such as Nickel, poison ivy and rubber, have been identified (1993 Immunology, 3rd edition, Published by Mosby, Editors: E. Roitt, J. Brostoff and D. Male).

5) In cancer:

evidence has been provided for antigen specificity of tumor infiltrating T lymphocytes. An example of such antigens has been described in 1993 J. Immunol. 151, 3719-3727.

The means for plating the human T cell lines can be chosen from a relatively large number of devices which can be operated by the person skilled in the art.

As for the T cell stimulating agent, it can also be chosen from a wide variety of available compounds. What is required is that the T cell stimulating agent be sufficiently potent to stimulate the development of T cells plated out at very low cell densities. Available compounds include those referred to above such as PHA. However, the person skilled in the art may select other stimulating agents that would

provide enhanced growth of T cells plated out at low densities.

In situations where the specific T cell lines can be readily identified from the biological sample or where it is required to have specific T cell monoclones to antigens which are different from one individual to another, the antigen is not an integral component of the kit. In this situation, the antigen-specific T cell lines are developed from biological samples which are related to the condition to be treated. As mentioned previously, in autoimmune diseases, patient peripheral blood lymphocytes are used, in tumor specific lymphocytes, cells obtained from excised tumors are used, in rheumatoid arthritis, cells found in the synovial fluid of the joints are used and in multiple sclerosis, cells found in the cerebro-spinal fluid are used.

Therapeutic formulation and administration of the T cell populations

The therapeutic use of the T cell populations of the present invention in the treatment of diseases or disorders can be accomplished by those skilled in the art using known principles of diagnosis and treatment. One important criterium is that the T cell clone population selected must have good growth characteristics, which permits large scale expansion of the clones to a sufficient amount that can range between 1×10^6 and 1×10^8 cells per clone.

Pharmaceutical compositions are prepared using inactivated cells or by combining inactivated cells to the appropriate carrier, which itself can be an immunological adjuvant. These compositions can be administered by any means that achieves the intended purpose. For example, administration may be subcutaneous, intravenous, intradermal, intramuscular or intraperitoneal.

The amount of cells administered as well as the frequency of administration is dependent upon the age, sex, health and weight of the recipient as well as the nature of the effect desired. Generally speaking, between 1 x 10^5 and 5 x 10^7 cells can be injected in at least 2 inoculations. The amount of cells administered should be sufficient to induce a substantial proliferative response to the vaccine preparation, preferably after the second inoculation.

For example, a pool of 10^7-1.5 x 10^7 irradiated cells can be prepared as a vaccine and injected subcutaneously. The selection of the amount of cells for vaccination can be made on the basis of an effective dose in humans such as described in Zhang et al. Science Vol 261, p. 1451-1454 (1993) or on the basis of an appropriate animal model such as the model described by Ben-Nun et al. in 1981, Nature 292, 60-63.

The number of inoculations necessary to induce the appropriate proliferative response against a particular vaccine clone or a mixture of clones can vary depending on the type of disease, disease state and the immunological state of the patent. Generally, for autoimmune diseases, at least two inoculations of 10^7-1.5 x 10^7 irradiated cells administered at 2 to 4 months intervals is sufficient to generate the appropriate response. In some situations, the number of inoculations needed can be higher dependent on the short and long term immune response of the patient to the specific T-cell vaccine and the antigen specificity of other possible pathogenic T-cells involved in the disease mechanism.

The response of the patient to the treatment is evaluated by analyzing the proliferation of anti-clonotypic T cells in patients injected with the T cell population of the invention. Briefly, peripheral blood mononuclear cells are isolated from the patient at different intervals following inoculation and plated

out for stimulation to the targeted antigens. If the patient has responded to the treatment, specific regulatory T cells are detectable in the patient. Normally, either CD4+ or CD8+ cell lines are stimulated by the inoculates. However, it is possible that other T cell populations are also induced by the vaccination, not only by exhibiting an inhibitory effect toward the vaccination product but also by driving the regulation network to enhance the suppression.

Diagnostic kit

The T cell monoclone population of the present invention can also be used in the diagnosis of conditions which result from the pathogenic role of these cells.

When it is necessary to provide a diagnosis for a patient suspected of suffering from a particular condition, a biological sample, or a lysate thereof, taken from the patient, is immobilized on a solid support. The presence of a particular pathogenic T cell is then determined by applying a monoclonal antibody directed against a recognized shared sequence of the T-cell receptor. Identification can be performed by various immunostaining techniques such as ELISA and flow cytometry, a well known procedure to those skilled in the art.

The diagnostic kit of the invention therefore comprises a solid support on which the biological sample can be deposited and the relevant T-cells immobilized. It also includes means for at least immobilizing the sample cells on the support. Among the means that can be used to fix the sample cells on the support, one may mention the attachment of the cells on ELISA plates with antibodies (see for example Lymphocytes: a practical approach, Ed. Klaus GGB, pp. 48-54 1987 IRL Press, Oxford, Washington D.C.) or

chemical cross-linking (see for example 1990 Anticancer research 10, 271-278).

The kit also comprises a monoclonal antibody or monoclonal antibodies to a specific T cell membrane receptor recognizing one of the antigens associated with the condition to be diagnosed. The antibodies can be obtained by methods known to those skilled in the art. See for example Kohler and Milstein, Nature 256: 495-497, 1975 and US patent 4,376,110. Such antibodies can be of any immunoglobulin class but are preferably of the IgG class. Antibodies can also be prepared from polyclonal antiserum taken from animals immunized with the human T cell monoclone population of the present invention and subjected to various purification techniques known by those skilled in the art. The antibodies used can be labelled with an enzyme, a fluorescent dye or a cheminoluminescent label as is well known to those skilled in the art. Alternatively, the antibodies can be labelled with a DNA fragment that can be amplified by PCR as has been described previously (Sano T et AL. Science, Vol. 259, p. 120-122, 1992).

Monoclonal antibodies of animal origin or fragments thereof or recombinant antibodies containing the antigen binding region of the original antibody can be "humanized" by linking a cDNA molecule encoding the region of the monoclonal antibody to DNA encoding the human constant region, using various approaches described for example in US patent 4,816,567, European patent publication EP 125023, EP 171496 and EP 173494 and PCT publication WO 8601533 and WO 8602671.

An example of the diagnostic kit of the invention is one to be used in the diagnosis of multiple sclerosis. Monoclonal antibodies to shared T cell monoclone receptors specific to immunodominant regions of MBP (residues 84-102 and 143-168 for example) are prepared and fixed on an appropriate

support. A biological sample taken from a patient suspected of suffering from multiple sclerosis is then contacted with the support. The positive binding of T cell receptors to the support indicates the presence of T cells specific for immunodominant MBP epitopes in the biological sample.

DESCRIPTION OF A PREFERRED EMBODIMENT OF THE INVENTION

1. MBP-specific T cell monoclone.

a. Generation and characterization of MBP-specific T cell lines.

To generate MBP-specific T cells from peripheral blood, fresh blood samples were obtained by venipuncture and diluted with an equal volume of RPMI medium (GIBCO). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient separation (Zhang et al., Cell. Immunol. 139, 118, 1992). This method comprises following steps : diluted blood is overlaid on Ficoll and centrifuged at 1,800 rpm for 20 min. Subsequently the PBMC are washed three times and resuspended to a homogeneous suspension. PBMC are then plated out by limiting dilution (Zhang et al., Ann. Neurol. 32,330, 1992) at 200,000 cells, 100,000 cells and 50,000 cells per wells (60 wells for each cell concentration) in U-bottomed microwell plates, consisting of 96 wells of 200 µl content/plate) (Costar, Cambridge, USA). To each well, 100,000 autologous irradiated (8,000 rads) PBMC are added as a source of antigen-presenting cells (APC) in the presence of 40 µg /ml of human MBP.

Human MBP is extracted from the white matter of human brain tissue and purified by column chromatography (Hashim et al., J. Neurosci. Res. 16, 467, 1986). These conditions were optimized, in a set of experiments involving more than 50 clinical blood samples, to give a maximal T cell response. Cultures were maintained in an incubator conditioned with 5% CO2

and 37°C for 7 days. After 7 days, cultures were restimulated with 100,000 /well irradiated autologous PBMC pulsed with MBP. Pulsing of PBMC was carried out by incubating PBMC with 100 μg /ml of MBP at 37°C for four hours. Free MBP was washed away prior to irradiation of the cells.

Selection of MBP-specific T cell lines was performed at Day 12 and Day 14 in a proliferation assay. Each culture was split into four aliquots (approximately 10^4 cells per aliquot) and cultured in duplicates in the presence of 10^5 autologous PBMC pulsed or non pulsed (control) with MBP for 72 hours. 1 μCi/well of ^{3}H-thymidine was added during the last 16 hours of culture and the cells were collected with the use of a cell harvester (Betaplate 1295-004, Pharmacia). Tritiated thymidine uptake was measured in a beta scintillation counter (Betaplate 1205, Pharmacia).

The frequency of MBP-specific T cells was calculated according to the Poisson statistics (Lefkovits et al. eds. Limiting dilution analysis of cells in the immune system. Cambridge, Cambridge University Press, 1979; Fey et al. J. Exp. Med. 158, 40, 1983). Briefly, a culture was scored positive if its mean CPM was greater than 1,000 and if the CPM were at least three times higher than the control CPM, a frequency of positive wells was obtained at each cell concentration. Estimation of the frequency of growth-positive T cells or antigen-specific T cells was then done by applying the Poisson Formula : $Fr = (u^r/r!) \times e^{-u}$, where Fr is the probability of obtaining r specific T cells in a well when the number of PBMC per well is u at a given concentration. The fraction of negative wells is given by $F_0 = e^{-u}$. When $u = 1$, $F_0 = 0.37$. Therefore, theoretically, when the average number of responding T cells per well is one, 37% of the wells will be scored as negative. Extrapolation to this point

in limiting dilution gives a number of cells, the reciprocal of which represents the frequency of the antigen-specific T cells in question. MBP-specific T cells occur at an estimated frequency between 10^{-7} and 10^{-6} in peripheral blood lymphocytes both in patients with MS and controls (Zhang et al., Ann. Neurol. 32, 330, 1992; Ota et al., Nature 346, 183, 1990). Culture medium used was RPMI 1640 supplemented with 10% autologous serum (heat-inactivated at 56°C for 30 min.), 2 mM L-glutamine, 50 µg/ml gentamicin, (Gibco, Life Technologies), and 10 mM Hepes buffer (Flow Laboratories, Belgium).

Selected MBP-specific T cell lines were plated out at 10,000 cells per well and restimulated with irradiated autologous APC pulsed with MBP. 7 days later, the cell lines were re-examined for their specific proliferation in response to MBP in a proliferation assay (described above). An example is given in Table I to illustrate the procedure. MBP-specific T cell lines were further examined for phenotype expression and reactivity to the MBP fragments and synthetic peptides as shown in Figure 1A-1B. To define the reactivity to fragments and peptides of human MBP, 10^4 cells of each MBP-specific T cell line were cultured with 10^5 irradiated autologous PBMC or EBV-transformed B cells pulsed with respective peptides. To prepare antigen-pulsed APC, PBMC or B cells were incubated with 2 to 5 µg/ml of a peptide or a peptide mix for four hours and washed two times before irradiation. Specific proliferative response to a fragment or peptide was measured in a proliferation assay.

Our data have revealed that T cell responses to MBP in humans are restricted to the CD4 phenotype and directed predominantly to two immunodominant epitopes on MBP. One is located within the 84-102 region and the other resides in the 149-170 region

(Reviewed in Zhang et al., Intern. Rev. Immunol. 9, 183, 1992). Reactivity to these two immunodominant epitopes accounts for more than 60% of the T cell responses to MBP (Zhang et al., Ann. Neurol. 32, 330, 1992; Ota et al., Nature 346, 183, 1990; Pette et al., Proc. Natl. Acad. Sci. 87, 7968, 1990).

Table I shows an example of a general scheme used to establish MBP-specific T cell lines from PBMC.

Figure 1 illustrates the reactivity pattern of a panel of MBP-specific T cell lines to three MBP fragments, spanning 1-38, 45-89 and 90-170 regions of human MBP (Figure 1A) and to synthetic peptides overlapping the 84-171 region of MBP (Figure 1B).

b. Single-cell cloning of MBP-specific T cells:

It has been problematic to clone out a true MBP-specific T cell clone owing to a limited source of autologous antigen-presenting cells (APC), a low cloning efficiency and the induction of T cell tolerance during the MBP stimulation process (LaSalle et al., J. Exp. Med. 176, 177, 1992). Therefore, cloning of MBP-specific T cell lines by repeated MBP stimulation in the presence of APC usually requires a seeding concentration of more than one cell per well. As a result, a resultant "clone" preparation is often contaminated with unwanted T cells. This contamination can be detected by the expression of TCR Vβ gene usage. A true clone usually gives rise to a single expression of TCR Vβ gene when tested with a panel of TCR Vβ gene primers by polymerase chain reaction (PCR) while multiple Vβ gene usages in a clone preparation indicates oligoclonal contamination. An example of such contamination is given in Figure 2 (panel A and panel B), which shows oligoclonality after cloning at 3 cells per well. This oligoclonality is most likely caused by contaminating T cells present in an original T cell line preparation. This can be further confirmed by Poisson statistics (see Lefkovits et al. eds. Limiting

dilution analysis of cells in the immune system. Cambridge, Cambridge University Press, 1979). Furthermore, these MBP-specific contaminated T cell "clones" display poor growth characteristics and frequently loose their antigen specificity. It, therefore, is extremely troublesome to maintain these "clones" in a long-term culture to reach high enough amounts of cells which are required for therapeutic use.

To cope with these problems an alternative cloning procedure was developed. In this method, PHA, a potent T cell stimulating agent, is used to clone MBP-specific T cell lines at very low cell densities. MBP-specific T cells are plated out at 0.1 cell and 0.3 cell per well in the presence of irradiated autologous or allogeneic PBMC and PHA at 0.2 to 10 µg/ml. Cultures are refed with fresh culture medium containing rIL-2 (5 units /ml) every three days. At Day 14, growth-positive clones (usually 6% - 10% positive rate) are tested for their specific response to MBP, as described above. MBP-specific T cell clones derived from this cloning procedure are highly proliferative to MBP and other T cell stimuli, including anti-CD3 antibodies (Weber et al., J. Immunol. 135, 2337, 1985), PHA, ConA and IL-2 and can be further expanded to more then 10^7 - 10^8 cells by alternate stimulation with MBP and PHA.

This method has many advantages over conventional cloning methods by MBP stimulation, including (1) higher cloning efficiency, (2) improved growth characteristics, which allow for a large scale expansion of the clones with PHA or MBP stimulation, (3) maintenance of MBP specificity after repeated expansions and (4) monoclonality, confirmed by a single TCR Vβ gene expression (Figure 4, panels C, D and E).

Figure 2 represents the PCR analysis of TCR Vβ gene usage of MBP-specific T cell clones cloned by repeated MBP stimulation (panel A-B) and by PHA

stimulation (panel C-D). Panel E represents single Vβ gene usage of a typical TIL clone cloned by the PHA method. The Vβ genes of the clones were first amplified by each of 20 family-specific primers with a standard PCR technique (35 cycles) and a particular Vβ gene product was then hybridized with a specific probe in a Southern blot analysis. An amplified TCR Vβ gene product(s) is indicated, along with molecular weight markers. Cβ refers to constant β gene products.

Figure 3 represents the comparison of cloning efficiency of MBP-specific T cell lines by PHA and MBP stimulation (a representative experiment). A MBP-specific T cell line (stimulation index 11.2) was cloned by limiting dilution at indicated cell concentrations and stimulated by MBP (left panel) or PHA (right panel) in the presence of autologous PBMC. Data are given as frequencies of growth-positive wells (open circles) and MBP-specific T cell clones (closed circles). The frequency of MBP-specific T cells was estimated by the Poisson probability to be 1/250 by MBP cloning and 1/5 by PHA cloning.

c. Vaccination procedure.

Selection of MBP-specific T cell clones for T cell vaccination is based upon two characteristics :

a. The peptide reactivity to the two immunodominant epitopes on the MBP molecule or to another epitope(s) predominantly used in that particular individual. It has been well documented that T cell recognition to MBP is predominantly directed at the 84-102 region and the 149-170 region of MBP (Zhang et al., Cell. Immunol. 129, 189, 1990; Zhang et al. Ann. Neurol. 32, 330, 1992; Ota et al. Nature, 346, 183, 1990). Although the encephalitogenic epitopes in humans remain unclear, it can be extrapolated from animal studies that immunodominant determinants are likely to have encephalitogenic properties (Vandenbark

et al., J. Immunol. 135, 229, 1985; Zamvil et al., J. Exp. Med. 162, 2107, 1985).

b. The second criterion is that the T cell clones selected must have good growth characteristics, which permit large scale expansions of the clones to a sufficient amount ($3x10_7$-6 x 10^7) for a total of at least two inoculations.

T cell clones are activated with MBP-pulsed autologous APC four days prior to inoculation and tested for common bacterial and viral contaminants (Hafler et al., Clin. Immuno. Immunopath. 62, 307, 1992). Cells are then washed three times with sterile PBS (filtered through a filter with 0.22 μm pore size) and irradiated at 8,000 rads. For each immunization, a pool of 10^7 - 1.5 x10^7 irradiated cells of at least two different MBP-specific T cell clones are prepared in 1 ml PBS as a vaccine and injected (5 x 10^6 cells in 0.5 ml of PBS per arm) subcutaneously. Selection of this amount of cells for vaccination is calculated on the basis of an effective dose in EAE (Beraud, in Edelson ed. Antigen and clone-specific immunoregulation, Ann. NY. Acad. Sci. 636, 124, 1991). The subcutaneous route of injection is chosen as experiments performed in rats showed that subcutaneous injection is as effective as intravenous injection (I. Cohen, unpublished data).

d. <u>In vivo induction of anti-clonotypic T cells:</u>

Table II shows the clinical data of six patients with MS who participated in the trial and the fine specificities of the MBP-specific T cell clones used for vaccination.

Table II represents the peptide reactivity of MBP-specific T cell clones used as inoculates. MBP-specific T cell lines were generated from peripheral blood of the patients, as described above and cloned at 0.3 cell per well by limiting dilution with 10^5 irradiated autologous feeders and PHA (2 μg /ml).

Cultures were refreshed with culture medium containing 5 units rIL-2/ml every three days. After 12 - 14 days, growing clones were examined for their reactivity to three fragments of MBP, covering 1-37, 45-89 and 90-170 regions of MBP (provided by Dr. SH Chou) and subsequently tested with 11 peptides of MBP (provided by Dr. D. Hafler). 10^4 cells of each clone were cultured with 10^5 irradiated autologous APC per well, to which 10 µg ml of each fragment or 2 µg /ml of each peptide was added. Cells were cultured for 72 hours and pulsed with [3H]-thymidine during the last 16 hours of culture and harvested (Betaplate 1295) to measure tritiated thymidine uptake. The same procedure was used in other proliferation assays mentioned elsewhere in this patent application.

Experiments were designed to follow-up T cell responses to the inoculates as compared to PHA-induced autologous T blasts. PHA-induced T blasts were prepared concurrently with MBP-specific T cell clones in order to parallel the cell growth cycle. To this end, freshly isolated PBMC were cultured for four days at 10^6 cells /ml in the presence of 2 µg /ml PHA. Cells were washed three times prior to use. As shown in Figure 4, all six patients developed a substantial proliferative response to the autologous vaccine preparation especially after the second inoculation. These responses were accompanied by a limited reactivity to the T blasts. The frequency analysis of the MBP-specific T cells revealed a progressive decline of circulating MBP-specific T cells, notably after the second inoculation. The decrease in the frequency of MBP-specific T cells was antagonistically correlated with the magnitude of the anti-clonotypic responses (Figure 4). The frequency fell below the detectable limit of our assay in five out of six recipients at the end of the clinical trial. MBP-specific T cells in patient HM could still be detected after the third vaccination, but at a five

fold lower frequency (1.1×10^{-7}) than the pre-vaccination value. By striking contrast, the frequency of TT-specific T cells remained unchanged in all recipients while the frequency of MBP-specific T cells in two non-recipient patients (parallel controls) also remained unchanged (Figure 5), which is compatible with a specific down-regulation of MBP-specific T cells, suggesting that these MBP-specific T cells were either eliminated or were non reactive to MBP.

Figure 4 shows the proliferative responses to the inoculates and control T cells and the changes in the frequency of MBP-specific T cells before and after each inoculation. The assays were performed before vaccination and at Day 3, Week 1, Week 2, Week 4, Week 6 and Week 8 after each inoculation. Fresh peripheral blood mononuclear cells (PBMC) were isolated and 5×10^4 cells /well were cultured in triplicates with 5×10^4 irradiated inoculates or autologous PHA-induced T blasts prepared concurrently for 72 hours. As a control, PBMC and irradiated inoculates or T blasts were cultured alone. Cell proliferations were measured by proliferation assays as mentioned above. Data are given as stimulation indices defined as the mean counts per minute (CPM) of PBMC plus irradiated inoculates or T blasts / the sum of CPM of PBMC cultured alone and CPM of irradiated inoculates or T blasts cultured alone. The frequency of MBP-specific and TT-specific T cells was analyzed before vaccination and after each inoculation. PBMC were plated out at 2×10^5 cells and 10^5 cells per well for MBP stimulation (40 µg /ml MBP) or plated out at 2×10^4 and 10^4 cells per well for TT stimulation (2.5 Lf TT /ml), respectively (60 wells for each concentration). The concentration range was predetermined to allow a sensitive detection. Cultures were then restimulated with MBP- or TT-pulsed PBMC as a source of APC and rIL-2 was added at 5 units /ml. After one week, each culture was split and tested for

specific proliferation to MBP or TT in a proliferation assay. A T cell line was defined "specific" when the ratio of the CPM of wells containing MBP- or TT-pulsed APC / CPM of control wells exceeded 3 and if Δ CPM was larger than 1,000. The frequency of antigen-specific T cells was estimated by dividing the number of specific wells by the total amount of PBMC plated out.

Figure 5 represents the relationship of changes in the frequencies of T cells reactive to MBP, TT and inoculates in recipients (GE and CW) and non-recipients (AH and GC). The frequency analysis of MBP- and TT-specific T cells is described above. To estimate the frequency of T cells responding to the inoculates, freshly isolated PBMC were plated out at $4x10^4$ and $2 x10^4$ cells per well and cultured with $4x10^4$ irradiated inoculates. After 7 days, cultures were restimulated with the irradiated stimulator (vaccine) and supplemented with rIL-2 (5 units /ml). At Day 14, 50% of each culture was taken out respectively and irradiated at 8,000 rads. Cells were then split into four aliquots and added in duplicate to culture wells containing 10^4 inoculates or TT-specific T cells and 10^5 irradiated APC pulsed with MBP or TT in proliferation assays to measure their inhibitory effect. The inhibition was measured as 1 - (proliferation in the presence of irradiated responding T cells as inhibitor / proliferation in the absence of the inhibitor) x 100%. Cultures exerting more than 60% inhibition on the proliferation of inoculates were considered as responding cell lines. The frequency was estimated by dividing the number of responding wells by total PBMC plated out($6 x10^4$ cells).

Based upon the observed T cell proliferative responses to the inoculates, experiments were designed to isolate responding T cells when the responses to the inoculates reached a peak level. To this end mononuclear cells were derived after the second or

third inoculation and were co-cultured with irradiated autologous MBP-specific T cell inoculates as stimulators. The cultures were re-challenged with the same irradiated T cell preparation. Selection of responding cell lines was based upon specific inhibition (> 70%) on proliferation of the inoculates to MBP (see Figure 5 legend). Specific suppressor T cells were detectable in all three recipients tested with estimated frequencies of 0.2 $x10^{-6}$ (BC), 2.3 $x10^{-6}$ (CW) and 5.2 $x10^{-6}$ (GE) but not in two non-recipient control patients (Figure 5). 24 short-term cell lines were selected from two recipients CW and GE for further characterization to define their phenotypic profile and reactivity. Our data revealed that all the cell lines expressed the CD3 phenotype and the $\alpha\beta$ T cell receptor. Twenty-two T cell lines were CD8+ and two were CD4+. The inhibition was not mediated by culture supernatants as they did not affect the proliferation of MBP-specific T cells. These inhibitory T cell lines were further examined for their functional properties and specific recognition of the autologous inoculates as compared to a tetanus toxoid (TT)-reactive clone. Figure 6A illustrates that both CD4+ (CW2F3) and CD8+ (CW1G9, GE1B3 and GE1D6) T cell lines were stimulated specifically by the autologous inoculates but not by the TT-reactive clone. They were potent inhibitors specifically for the inoculates (Figure 6B). With the exception of the CD4+ cell line, all three CD8+ lines were found to lyse the inoculates in a standard 4-hour chromium-release assay (Figure 6C) and this antigen-specific cytotoxicity could be blocked by the addition of a monoclonal antibody to MHC class I molecules (W6/32) but not by an antibody to the class II products (Figure 6D), indicating that the T cell recognition of the inoculates was restricted by MHC class I molecules. Similar results were obtained from seven other CD8+ cell lines. Thus, these T cell lines may be classified

as anti-clonotypic T cells because of their specific recognition of a clonotypic structure on the MBP specific T-cells in the inoculates (see Lamb et al., Nature 300, 456, 1982; Mohagheghpour et al., J. Exp. Med. 164, 950, 1986; Holoshitz et al., Science 219, 56, 1983). It is possible that the anti-clonotypic T cells we obtained represent only a part of the T cell populations induced by the vaccination since the selection was based on their inhibitory effect. Other responding T cells may act by driving the regulation network to enhance the suppression, as typically illustrated by anti-ergotypic T cells isolated from vaccinated experimental animals (Lider et al., Science 239, 181, 1988).

Figure 6 represents the functional properties of the anti-clonotypic T cell lines. Panel A, anti-clonotypic T cell lines, tested as responders, were plated out in triplicates at 2 x 10^4 cells /ml and cultured with 4 x 10^4 autologous inoculates or TT-specific T cells as stimulators, which were irradiated (8,000 rads) to prevent their own proliferation. The CPM of the irradiated stimulators did not exceeded 1,200. Panel B, anti-clonotypic T cell lines were irradiated and used as inhibitors. 10^4 cells were added in triplicates to wells containing 10^4 cells from the inoculates or TT-specific T cells and 10^5 APC pulsed with MBP or TT in a proliferation assay. The percentage of inhibition was calculated as specified in Figure 5. Panel C, the inoculates or TT-specific T cells were labeled with 200 μCi ^{51}Cr for 45 min., subsequently washed four times and used as target cells in a standard chromium-release assay. After four-hours of incubation, supernatants were harvested and the radioactivity was measured. The effector (anti-clonotypic T cells) to target (the inoculates and control T cells) ratio was eight. The maximum and spontaneous releases of chromium were determined in

wells containing detergent or medium alone. The percentage of specific cytolysis was calculated as ((experimental release - spontaneous release) / (maximum release - spontaneous release)) x 100. Panel D, three anti-clonotypic clones were tested for antibody blocking in a chromium release assay. The antibodies used were either directede against class I molecules (W6/32) or against class II molecules (HB55). AHF4.2 was a CD4+ cytotoxic T cell clone specific for MBP-pulsed target cells used as a control. Effector clones were pre incubated with indicated antibodies at 10 μg /ml for 30 min. before mixing with ^{51}Cr-labeled target cells. The effector to target ratio was eight.

e. Monitoring of clinical improvement and possible toxic effects induced by T cell vaccination

Monitoring for toxicity over the entire trial confirmed that this vaccination was safe as no side-effects were observed and no changes in the standard systematic toxicity tests were observed. There was no evidence for acute exacerbations after the vaccinations.

Administration of the vaccines induced substantial anti-clonotypic T cell responses specifically to the vaccine clones, which were accompanied with a specific depletion of circulating MBP-reactive T cells in all six recipients. These responses were marked by a boosting effect with each vaccination (Figure 1). The in vivo depletion of MBP-reactive T cells appears to be the direct effect of anti-clonotypic T cells since the CD8+ anti-clonotypic T cell lines isolated from the vaccinated patients specifically lyse the autologous vaccine clones. The study has confirmed in a clinical setting that T cell vaccination can be used to boost clonotypic regulatory mechanisms in depleting pathologically relevant autoreactive T cells.

Fig. 9 represents the anti-clonotypic T cell responses to the vaccine clones and changes in the estimated frequency of circulating MBP-reactive T cells in six patients with MS, before and after each inoculation.

The responses to the vaccine clones were determined in proliferation assays, in which peripheral blood mononuclear cells (PBMC) were cultured with irradiated vaccine clones. The proliferative responses were calculated as stimulation indices (proliferation of PBMC in the presence of vaccine clones/the sum of spontaneous proliferation of PBMC alone and residual proliferation of irradiated vaccine clones). Data are given as mean stimulation indices of seven assays after each inoculation. The frequency of MBP-reactive T cells was estimated according to the method described in ref. 25. The frequency before vaccination is indicated on the lines, which ranges from 5.8×10^{-7} to 11.8×10^{-6} in these patients.

There are a number of issues that have emerged from the study. First, the clinical study has confirmed that clonally expanded MBP-reactive T cells in MS represent a dominant TCR repertoire and depletion of this population(s) eradicates the major responses to MBP. In this context, a question may be raised as to whether the depletion of a dominant TCR repertoire will lead to the display of a previously cryptic epitope(s) substitutive for the lost repertoire. Although MBP-reactive T cells have not been found in the vaccinated patients two years after vaccination using the whole MBP molecule as a probe, this possibility can not be ruled out as they may emerge after some time with a different label (different epitope reactivity and V gene usage). Furthermore, the study suggests that the anticlonotypic T cells recognizing MBP-reactive T cells are pre-existing and occur at a rather low frequency in MS patients prior to vaccination. The responses are

boosted by each inoculation and their frequency mounts typically to a ten-fold increase after the second and the third vaccination. Thus, it is important to further address the questions as to whether the anticlonotypic T cell responses are consistently low in MS patients and whether they are associated with hyperactivity of MBP-reactive T cells in the disease.

As for the molecular identity of the target sequence(s) that triggers the anticlonotypic T cells, at least tow variable regions have been mapped so far using a panel of $CD8^{+}$MHC class I-restricted anti-clonotypic T cells isolated from three vaccinated patients (Figure 10). One involves the CDR3 region characteristic for its unique junctional sequence of a given vaccine clone, as indicated by recognition of anti-clonotypic T cells to a target TCR sequence uniquely expressed on the immunizing T cells. The anti-clonotypic T cell clones with this recognition pattern responded specifically to the immunizing MBP-specific T cell clone but not to a total of 18 other autologous and MHC-matched allogeneic MBP-specific T cell clones not used for vaccination. The other pattern is associated with a clonotypic marker relatively conserved within the Vα region among autologous T cells. This is evident by their reactivity, in addition to the immunizing T cell clones, to other autologous and MHC-matched allogeneic MBP-specific T cells bearing the same Vα sequences. The CDR3 recognition pattern seems to be the dominant one and is highly specific for the immunizing clones. The other target sequence involved is likely to reside within the CDR2 or related regions and this recognition is less selective. In addition to the immunizing clones, the anti-clonotypic T cell lines of this recognition pattern affect autologous or MHC-matched T cells that have an unrelated specificity but bear the same Vα gene products T cell vaccination could be generalized using

a peptide(s) to a category of patients whose targeted autoreactive T cells share a common TCR structural feature. A more generalized form of T cell vaccination can depend on its simplified version that takes the advantage of using synthetic peptides or related T cell membrane fractions containing a desired target sequence(s).

The CDR2 region sequence is relatively conserved, implying that it is shared by a category of individuals. The Vα CDR2 sequences may have more limited heterogeneity as compared to its Vβ coutnerparts. Thus, to augment a CDR2-related clonotypic interaction, a library of "made-to-fit" peptides may be generated and a particular "off-shelf" peptide can be selected to attack a given CDR2 or a related sequence shared by the clonally expanded autoreactive T cells in a group of patients. In contrast, a CDR3 region sequence is known to be highly diverse from clone to clone. For a CDR3-restricted regulation, a potential use of similar strategy relies solely on the possibility that the target sequences of the CDR3 recognition pattern may display limited motifs within the V-D-J regions and these sequence motifs may constitute a common epitope(s) for clonotypic interaction. Indeed, such limited V-D-J sequence motifs have been identified among T cells specific for the 89-106 region (one of the immunodominant regions) of human MBP and these common motifs are rather consistent among 89-106 reactive T cells, irrespective of their host origins.

f. Cloning of anti-clonotypic T cells.

The same procedure as described sub b) for the cloning of MBP-specific T cells was applied for the cloning of anti-clonotypic T cells. Technically anti-clonotypic T cell lines as described sub d) were plated out at 0.1 cell and 0.3 cell per well in the presence of irradiated autologous or allogeneic PBMC and PHA at

2 µg/ml. Cultures are refed with fresh culture medium containing rIL-2 (5 units/ml) every three days. At Day 14, growth-positive clones (usually 8% - 10% positive rate) are tested for their specific recognition and cytotoxic activity towards the inoculates. Anti-clonotypic T cell clones derived from this cloning procedure are highly proliferative to the irradiated inoculates and other T cell stimuli and can be further expanded to more than 10^7 - 10^8 cells by adding rIL-2 at each cell passage.

Table III illustrates a typical experiment in cloning of anti-clonotypic T cell lines.

2. Tumor-specific T cell monoclone.

a. Generation and characterization of tumor specific lymphocytes (TSL).

Tumors excised from patients were immediately transported from the hospital to the laboratory. They were then minced into 1-2 mm pieces and subsequently treated with an enzymatic solution containing hyaluronidase type V 0.01%, collagenase type IV 0.1% (Sigma, Vel, Belgium), DNase type I 0.002%, gentamicin 50 µg/ml and fungizone 250 ng/ml dissolved in RPMI 1640 medium (Gibco, Life technologies, Belgium). The mixture was incubated for 2-4 hours at 37°C or overnight at room temperature. It was then filtered through a sterile coarse wire grid, washed four times with RPMI 1640 medium, and resuspended in culture medium which was RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 µg/ml gentamicin, 250 ng/ml fungizone (Gibco, Life Technologies), and 10 mM Hepes buffer (Flow Laboratories, Belgium). rIL-2 was added at a final concentration of 200 U/ml (Eurocetus). 200 U/ml of IL2 obtained from Eurocetus equals to 5 U/ml obtained from Bochringer Manheim (Germany) (Zhang et al. J. Exp. Med. Vol. 179 1994) TSL were cultured in 24-well plates (Costar, ElsColab, Belgium) in 2 ml

aliquots the first 4 weeks and then after dividing the aliquots in two equal parts cultured in the absence or presence of oxidized PBL (see below) in 24-well or 12-well plates.

Expansion of tumor specific lymphocytes (TSL) in vitro is hampered by several factors, including the limited amount of lymphocytes that can be obtained from tumors, unknown target antigens and a limited supply of antigen-presenting cells (APC) which are generally believed to be essential in the classical way of T cell stimulation and expansion. In approaching these difficulties, we have used surface-oxidized allogeneic PBL to stimulate the TSL periodically in the presence of rIL-2 (200 IU/ml) (Chin, Y. et al. Anticancer Res. 12, 733, 1992). TSL derived from 22 (out of 23) tumor specimens could be expanded with 20 -10^7 fold increases over 6 - 16 weeks, to a sufficient amount of 10^9 -10^{11} cells for adoptive immunotherapy. In contrast, only 2 - 100 fold increases were observed in six tumor specimens (out of 23) when 200 IU/ml rIL-2 was used only. The phenotypes, autologous tumor reactivity and cytolytic capability of TSL propagated with surface-oxidized stimulators were similar to those expanded in the presence of IL-2 alone. These data suggest that expanding TSL with surface-modified stimulator cells could be a useful alternative method to obtain large amounts of tumor specific cytolytic T cells for clinical immunotherapeutic use, irrespective of tumor-antigen stimulation and MHC restriction.

Oxidation of PBL was performed according to Novogrodsky and Fleischer. Briefly, irradiated allogeneic PBL ($4x10^7$-6 x 10^7 cells/ml) from normal subjects were incubated with galactose oxidase 0.05 U/ml (Sigma) and neuraminidase 0.02 U/ml (Boehringer Mannheim, Germany) in RPMI 1640 medium for 90 min. at 37°C and shaken at 15 min. intervals to prevent formation of clumps. The cells were washed three times

with RPMI 1640 containing 0.01 M galactose (Sigma) to block the residual effects of galactose oxidase. Oxidized PBL were added to TSL cultures mentioned above at a ratio of 5-10 oxidized PBL to 1 TSL. Cells were restimulated on a weekly basis with oxidized PBL in culture medium containing fresh rIL-2, and viable cell concentrations were returned to 0.5 x 10^6 cells/ml at each passage.

TSL cell lines were then characterized as to their proliferative response to autologous and allogeneic tumor targets and their cytotoxic activity against the tumor targets.

Figure 7 shows the proliferative response of TSL lines to autologous and allogeneic tumor targets. 10^4 cells of each TSL line were cultured in triplicate in the presence of 10^5 irradiated PBMC and autologous or allogeneic tumor cells, respectively. Microcultures were then pulsed with 1 μCi of [^{3}H]-thymidine (Radiochemical Center, Amersham, England) per well 4 hours prior to harvesting and thymidine uptake was measured by liquid scintillation counting.

Figure 8 represents the cytotoxic activity of the TSL lines against autologous and allogeneic tumor cells, NK-sensitive K562 cell line, and NK-resistant Daudi cell line. Target cells were labeled with 200 μCi $^{51}Cr(Na_2Cr_3O_4$, Amersham, England) for 60 min. at 37°C and washed four times with medium. Target cells were reincubated for another 30 min. and washed twice before use. 5 x 10^3 labeled target cells were incubated with TSL in 96-well plates in triplicate at various effector:target ratios in a total of 200 μl volume. Supernatants were harvested with a Skatron-Titertec system after 4 hour incubation at 37°C and the radioactivity was counted in a gamma counter. The maximum release and spontaneous release of chromium were measured in wells containing target cells in the presence of detergent or medium alone.

The specific release was calculated as

$$\% \text{ specific lysis} = \frac{\text{exp.release} - \text{spon.release}}{\text{max.release} - \text{spon.release}} \times 100\%$$

b. Single cell cloning for tumor infiltrating T cell lines specific for tumor antigens

The same cloning procedure is applicable for establishing tumor-specific TIL clones for therapeutic use. As described in 1-b, TIL lines are cloned by limiting dilution at 0.1 cell, 0.3 cell and 1 cell per well in the presence of irradiated allogeneic PBMC and 2 μg PHA/ml. Cultures are refed with fresh culture medium containing r-IL-2 (5 units/ml) every three days. At Day 14, growth-positive clones (usually 8 - 10% positive rate) are tested for their specific cytotoxic activity against autologous tumor targets in a standard chromium-release assay as described above. Specific clones derived from this cloning are highly proliferative to autologous tumor cells and to other T cell stimuli and can be further expanded to more than 10^9 - 10^{11} cells, required for adoptive immunotherapy, by adding rIL-2 at each cell passage. TCR Vβ gene usage of a typical TIL clone is given in Figure 2 (panel E).

3. Foreign antigen specific T cell monoclone.

a. Generation of T cell lines specific for foreign antigens

The procedure for the generation of MBP-specific T cells can be applied to other antigens as well. Tetanus Toxoid (TT) specific T cell lines were generated as described for MBP-specific T cell lines in 1-a. The concentration of Tetanus Toxoid antigen used was 2.5 Lf TT per ml.

b. Single cell cloning of T cells specific for foreign antigens.

Tetanus toxoid (TT) specific T cells were cloned with the procedure described in 1-b. Technically TT-specific T cells are plated out at 0.1 cell and 0.3 cells per well in the presence of autologous or allogeneic PBMC and PHA at 2 μg/ml. Cultures are refed with fresh culture medium containing r-IL-2 (5 units/ml) every three days. At Day 14, growth-positive clones (usually 8 - 10% positive rate) are tested for their specific response to Tetanus Toxoid. TT-specific T cell clones derived from this cloning are highly proliferative to Tetanus Toxoid and other T cell stimuli and can be further expanded to more than 10^7 - 10^9 cells by adding rIL-2 at each cell passage. This method has similar advantages over conventional cloning by TT stimulation as specified in 1-b.

The same procedures can be applied for the generation of T-cell lines and the isolation of T cell monoclones that are specific for other antigens such as allergens which are responsible for T cell mediated allergies. The expanded T-cell monoclones, specific for T-cell mediated allergies, can be used for the treatment of these allergies by applying the same vaccination procedure as specified in 1-c.

Table I Example of a typical generation of MBP-specific T cell lines from a patient with MS

experimental setup	time schedule	T cell line	stimulation index
200,000 cells /well	day 0		
100,000 cells /well			
restimulation	day 7		
addition of rIL-2	day 9		
proliferation assay	day 14	1E3	5.2
		1E4	9.6
		1E5	11.2
		2G5	12.6
		3F6	5.9
		3F7	3.8
restimulation	day 17		
addition of rIL-2	day 19		
proliferation assay	day 24	1E3	27
		1E4	132
		1E5	74.6
		2G5	43.7
		3F6	4.2
		3F7	120.3
single-cell cloning	day 27		

Table II Clinical data of the recipients with MS and fine specificity of the MBP-specific T cell clones used in the clinical trial.

patient	age/sex	diagnosis	EDSS	T cell clone	proliferative response (CPM incorporated x 10^3)				
					medium alone	MBP	p84-102	p143-168	p110-129
BC	43/M	chronic progressive	7.0	BC12	0.2±0.02	6.8±0.5	0.3±0.04	8.2±0.6	N.T
				BC-6	0.2±0.01	5.6±0.4	0.3±0.02	0.2±0.01	3.4±0.1
				IB7-E4	0.1±0.01	42.5±3.1	43.8±2.2	0.1±0.01	N.T
BR	31/F	relapsing remitting	1.0	BR-1	1.2±0.2	28.7±2.2	1.6±0.2	22.1±2.8	N.T
				BR-3	0.8±0.09	16.6±1.8	1.1±0.15	18.4±2.5	N.T
				IG7	1.7±0.1	52.3±4.8	1.3±0.1	47.4±5.2	N.T
RM	47/M	relapsing remitting	4.5	ID5	0.8±0.08	45.8±5.9	1.2±0.14	38.7±4.6	N.T
				HM-1	1.4±0.12	78.5±7.9	1.8±0.2	82.2±7.2	N.T
CW	46/M	chronic progressive	7.5	CW-5	0.1±0.01	17.8±1.6	0.2±0.01	0.4±0.03	0.2±0.02
				CW-10	0.1±0.01	5.4±0.6	0.1±0.01	7.8±0.8	N.T
				1E4	0.1±0.01	37.6±4.8	0.3±0.02	27.3±2.4	N.T
NF	46/F	primary progressive	4.5	C10	0.7±0.04	32.8±2.6	1.1±0.02	1.4±0.06	1.1±0.1
				IB3	1.1±0.1	13.1±1.2	0.7±0.05	1.3±0.08	1.8±0.2
GE	26/F	relapsing remitting	3.0	GE-2	1.4±0.1	20.1±1.8	11.2±0.8	1.1±0.1	N.T
				GE-3	1.6±0.1	42.2±2.3	23.6±2.1	2.2±0.1	N.T
				GE-4	2.8±0.3	37.0±3.2	24.8±2.2	1.9±0.2	N.T

TABLE III Cloning of anti-clonotypic T cell lines by the method of the invention

T cell line	growth-positive / total culture wells	specific clones / growth-positive	(% specific cytolysis)	
1F4	8/180	3/8	1F4-4	(64%)
			1F4-5	(70%)
			1F4-7	(68%)
1G9	5/180	1/5	1G9-1	(45%)
1B8	14/180	2/14	1B8-5	(82%)
			1B8-10	(86%)
2C7	4/180	1/4	2C7-4	(48%)
1D6	14/180	6/14	1D6-1	(91%)
			1D6-3	(89%)
			1D6-9	(100%)
			1D6-11	(77%)
			1D6-12	(69%)
			1D6-14	(94%)
1B2	8/180	5/8	1B2-2	(78%)
			1B2-3	(82%)
			1B2-5	(74%)
			1B2-7	(66%)
			1B2-8	(54%)
1B3	7/180	4/7	1B3-D4	(56%)
			1B3-E6	(43%)
			1B3-F8	(42%)
1E7	6/180	3/6	1E7-G6	(48%)
			1E7-F4	(52%)
			1E7-F10	(43%)

negative clones < 2-8%

Table IV Evidence for clonal expansion of MBP-reactive T cells in patients with MS

subject	No. of clones	peptide reactivity	DR restriction	Vβ usage	V-D-J DNA sequence pattern
MS-1	5	84-102(3/5) only MBP (1/5) only MBP (1/5)	DR2(5/5)	7.2 (3/5) 7.1 (1/5) 7.1 (1/5)	sequence pattern 1* (3/5) sequence pattern 2 (1/5) sequence pattern 3 (1/5)
MS-2	7	84-102 (7/7)	DR2 (7/7)	2.1 (6/7) 21 (1/7)	sequence pattern 1* (5/7) sequence pattern 2 52/7)
MS-3	15	84-102 (15/15)	DR2 (15/15)	17.1 (5/15) 17.1(6/15) 6.1 (3/15) 4.3 (1/15)	sequence pattern 1* (5/15) sequence pattern 2* (6/15) sequence pattern 3 (3/15) sequence pattern 4 (1/15)
MS-4	3	143-168 (3/3)	DR2 (3/3)	13.1 (3/3)	a single sequence pattern* (3/3)
MS-5	4	84-102 (4/4)	DR7 (4/4)	17.1 (4/4)	a single sequence pattern* (4/4)

34 independent MBP-specific T cell clones isolated from finve MS patmients were analyzed for their Vβ gene ussage and the V-D-J junctional DNA sequences by PCR techniques. The number of clones positively tested for the indicated parameters is given in parentheses. A predominant V-D-J sequence pattern(s) is indicated by the asterisk.

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CLAIMS

1. A population of human T cell monoclones which is highly proliferative in the presence of an antigen to which said human T cells are specific and which is characterized by its full biological purity in that it remains free of contaminating cells at all stages of subsequent culture development.

2. Human T cell monoclone population according to claim 1 characterized in that it gives rise to a single TCR V gene expression.

3. Human T cell monoclone population according to any one of the preceding claims characterized in that it possesses a unique TCR V-D-J DNA sequence.

4. Human T cell monoclone population according to any one of the preceding claims characterized in that it comprises cells of either the CD4 or the CD8 phenotype.

5. Human T cell monoclone population according to any one of the preceding claims characterized in that the antigen in the presence of which said population is proliferative is a tumor cell or an immunogenic portion thereof.

6. Human T cell monoclone population according to any one of claims 1 to 5, characterized in that the antigen in the presence of which said population is proliferative is an auto-antigen.

7. Human T cell monoclone population according to claim 6, characterized in that the auto-antigen is a Myelin antigen or an immunogenic portion thereof.

8. Human T cell monoclone population according to claim 7 characterized in that the Myelin antigen is selected from the group consisting of the Myelin Basic Protein (MBP), the Proteolipid Protein (PLP), the Myelin-Associated-Glycoprotein (MAG), the

Myelin-Oligodendrocyte-Glycoprotein (MOG) and/or a mixture thereof.

9. Human T cell monoclone population according to claim 8, characterized in that the Mielin antigen is an epitope of the 84-102 region or the 149-170 region of the amino acid sequence of Myelin Basic Protein.

10. Human T cell monoclone population according to any one of claims 1 to 4 characterized in that the antigen in the presence of which said population is proliferative is a foreign antigen.

11. Human T cell monoclone according to claim 10 characterized in that said foreign antigen is a Tetanus Toxoid antigen.

12. Human T cell monoclone population according to any one of claims 1 to 4 characterized in that the antigen in the presence of which said population is proliferative is an allergen that is mediating the allergy through T cells.

13. Human T cell monoclone directed to the human T cell monoclone according to any of the preceding claims.

14. Human T cell monoclone according to claim 13, directed to a Myelin Basic Protein-specific human T cell monoclone.

15. Human T cell monoclone according to claim 13, directed to a human T cell monoclone specific for the epitope of the 84-102 region or ghe 149-170 region of the amino acid sequence of the Myelin Basic Protein.

16. A method for the production of a population of human T cell monoclones which is highly proliferative in the presence of the antigen to which said human T cells are specific and/or any other T cell stimulating agent, and which is characterized by its full biological purity in that it remains free of contaminating cells at all stages of subsequent culture development, said method comprising:

(1) providing a human T cell line responsive to said antigen;

(2) single cell cloning said T cell line and stimulating the resulting T cell clone with a T cell stimulating agent in the presence of autologous or allogeneic feeder cells to produce populations of human T cell monoclones; and

(3) selecting the monoclone population having the desired TCR-specific characteristics.

17. A method according to claim 16 characterized in that said human T cell line is taken from peripheral blood lymphocytes (PBL)

18. A method according to claim 16 or 17 characterized in that T cell stimulating agent is selected from the group consisting of lectines, preferably PHA and/or ConA, lymphokines, preferably Interleukin-2 (IL-2) and/or a recombinant IL-2(r-IL2) mitogenic antibodies against CD3 and other cell surface molecules and/or a mixture thereof.

19. Process according to claim 18, characterized in that the T cell stimulating agent is PHA.

20. A homogeneous population of T cell receptors from human T cell monoclones according to any one of claims 1 to 12 or the antigen-specific portion thereof and/or a mixture of selected populations or portions.

21. A therapeutic agent for the treatment of autoimmune diseases, said therapeutic agent comprising an effective amount of a population or a mixture of selected populations of T cell monoclones according to any one of claims 1 to 10.

22. A therapeutic agent for the treatment of a T cell mediated allergy, said therapeutic agent comprising an effective amount of a population or a mixture of selected populations of T cell monoclones according to any one of claims 1 to 4 and 12.

23. A therapeutic agent for the treatment of infections and cancer, said therapeutic agent comprising an effective amount of a population or a mixture of selected populations of T cell monoclone according to any one of claims 1 to 6.

24. A vaccine composition for conferring upon humans active immunity against autoimmune diseases, said vaccine composition comprising an effective amount of a homogeneous population of T cell receptors according to claims 1 to 12 from human T cell monoclones or the antigen-specific portion thereof.

25. A vaccine composition for conferring upon humans active immunity against autoimmune diseases, said vaccine composition comprising a population or a mixture of selected populations of T cell receptors obtained from the population of human T cell monoclones according to claim 1 to 12.

26. A method for the treatment of a patient suffering from a condition associated with one or more antigens specific to said condition and obtainable from a biological sample of said patient, said method comprising:

vaccinating said patient with or adoptively transferring to said patient an amount of a human T cell monoclone population sufficient to generate the appropriate immune response to at least partially alleviate said condition, whereby said human T cell monoclone population is responsive to said one or more antigens and has a full biological purity in that it remains free of contaminating cells at all steps of culture development.

27. A method according to claim 26 wherein said condition is associated with one or more antigens specific to an infection.

28. A method according to claim 26 wherein said condition is associated with one or more antigens specific to an autoimmune disease.

29. A method according to claim 26 wherein said condition is associated with one or more antigens specific to a T cell mediated allergy.

30. A method according to claim 26 wherein said condition is associated with one or more antigens specific to a cancer.

31. A kit for the identification of those human T cell monoclones which are highly proliferative to an antigen of the type which may be held responsible of a particularly diagnosed disease and for the subsequent preparation of a population of said identified human T cell monoclones, said kit comprising:

(1) an antigen specific to the diagnosed disease in sufficient amounts to generate cell lines responsive to said antigen from a biological sample;

(2) means for plating said human T cell lines at very low cell densities; and

(3) a T cell stimulating agent for growing said low density human T cells.

32. A kit according to claim 31, further comprising protocols and essential reagents for the characterization of said T cell monoclones.

33. A kit according to claim 31 or 32 wherein said antigen is Myelin Basic Protein.

34. A kit according to claim 31 OR 32 wherein said antigen is an epitope of the 84-102 region or the 149-170 region of the amino acid sequence of Myelin Basic Protein.

35. A kit according to claim 31 or 32 wherein said antigen is selected from the group consisting of Collagen type II, Heat shock Protein or Superantigens.

36. A kit according to claim 31 or 32 wherein said antigen is a Proteolipid Protein.

37. A kit according to claim 31 or 32 wherein said antigen is Glutamic acid decarboxylase.

38. A kit according to claim 31 or 32 wherein said antigen is selected from the group consisting of Nickel, poison Ivy and rubber.

39. Use of one or more selected population(s) of antigenic human T cell monoclones according to claims 1 to 2 for the treatment of infectious diseases, autoimmune diseases, T cell mediated allergies or cancer.

40. Use of a homogeneous population or a mixture of selected homogeneous populations of T cell receptors from human T cell monoclones according to any one of claims 1 to 12 or the antigen-specific portion thereof for the treatment of infectious diseases, autoimmune diseases, T cell mediated allergies or cancer.

41. Use of a population or a mixture of selected populations of human T cell monoclones according to any one of claims 1 to 12, for the production of a pharmaceutical composition intended for the treatment of infectious diseases, autoimmune diseases, T cell mediated allergies or cancer.

42. A diagnostic kit comprising an appropriate solid support for immobilizing a biological sample containing a specific T cell responsive to an antigen, means for at least immobilizing said specific T cell on said support and an antibody to a T cell monoclone receptor that binds the antigen associated with or specific to the condition to be diagnosed.

43. A diagnostic kit according to claim 42, wherein said antibody is a monoclonal antibody.

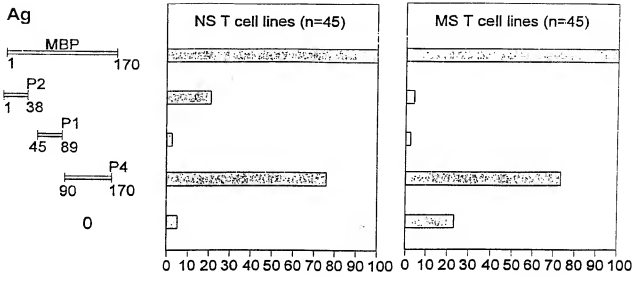

Figure 1-A

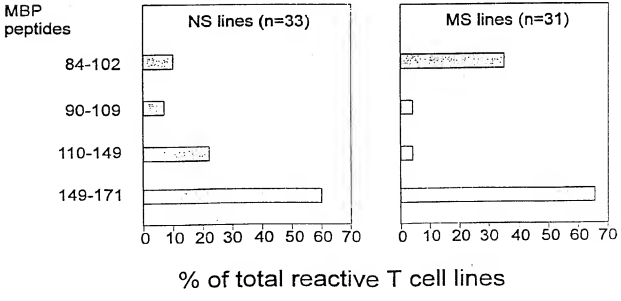

Figure 1-B

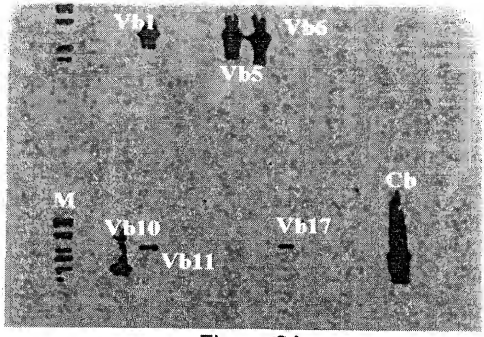

Figure 2A

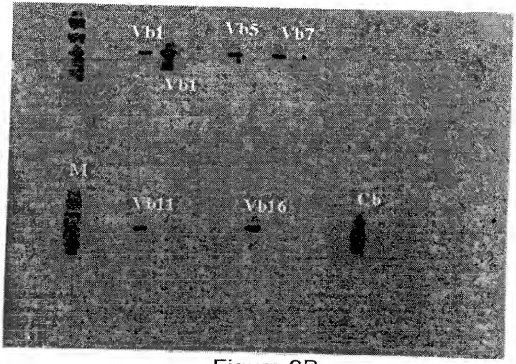

Figure 2B

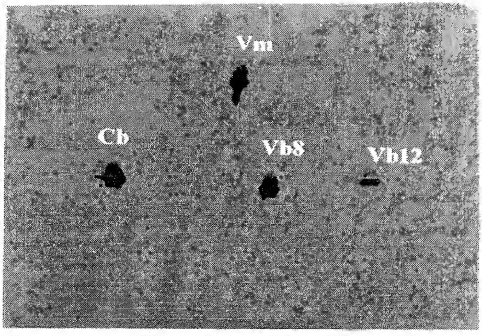

Figure 2C

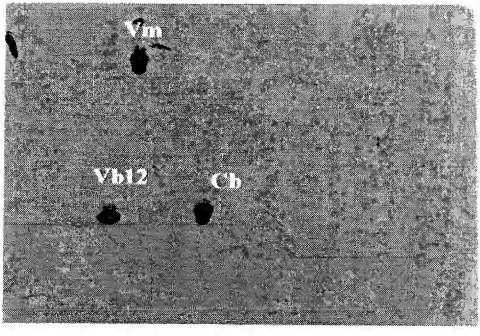

Figure 2D

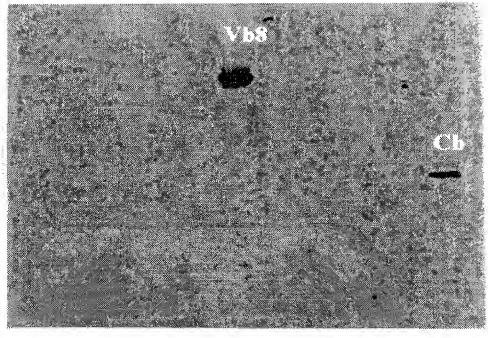

Figure 2E

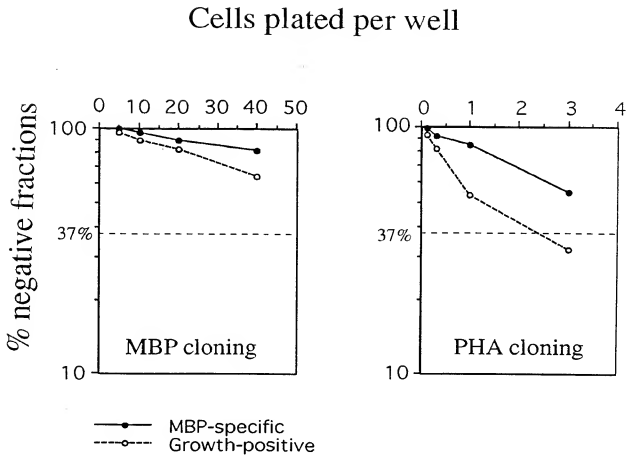
Cells plated per well
% negative fractions
0 10 20 30 40 50
100
37%
10
MBP cloning
0 1 2 3 4
100
37%
10
PHA cloning
MBP-specific
Growth-positive

Figure 3

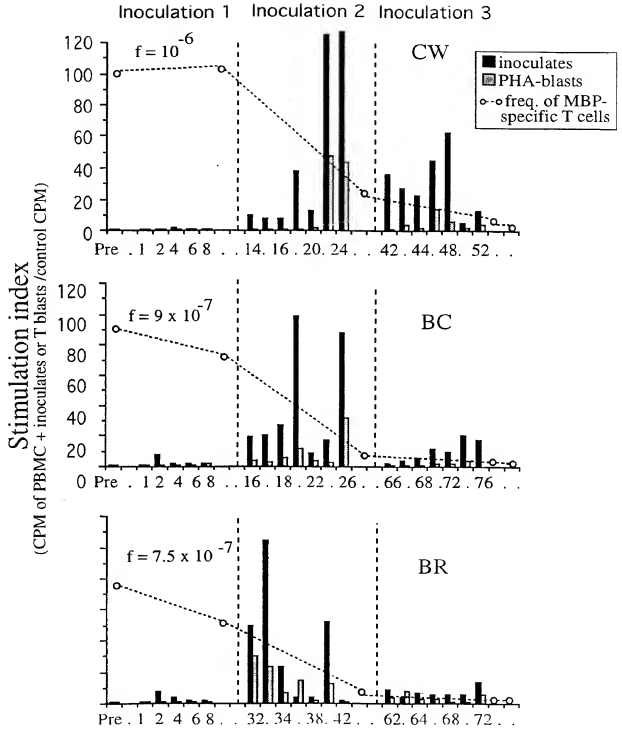
Inoculation 1
Inoculation 2
Inoculation 3
f = 10-6
CW
inoculates
PHA-blasts
freq. of MBP-specific T cells
Stimulation index
(CPM of PBMC + inoculates or T blasts /control CPM)
Pre . 1 2 4 6 8 . . 14. 16 . 20. 24 . . 42 . 44. 48. 52 . .
f = 9 x 10-7
BC
0 Pre . 1 2 4 6 8 . . 16 . 18 . 22 . 26 . . 66 . 68 .72 . 76 . .
f = 7.5 x 10-7
BR
Pre . 1 2 4 6 8 . . 32. 34 . 38. 42 . . 62. 64 . 68 . 72 . .
Time schedule (in weeks)

Figure 4A

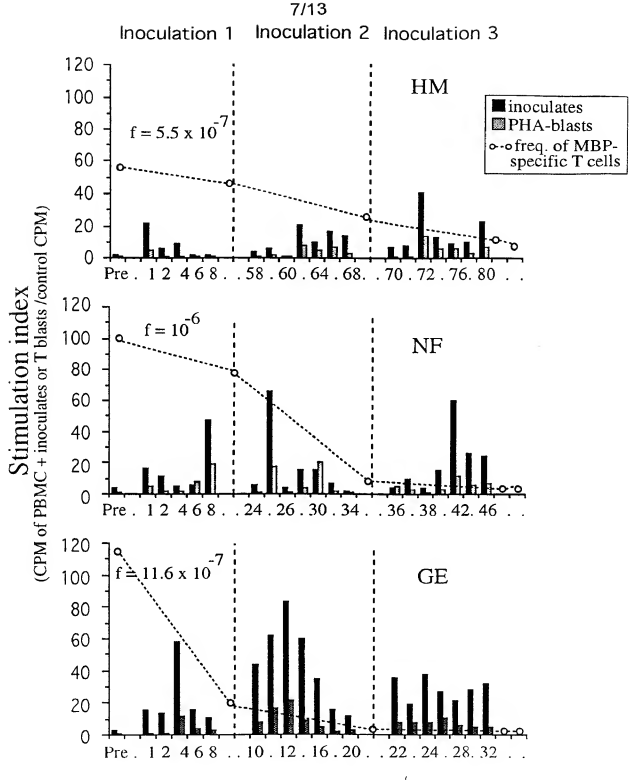
Inoculation 1
Inoculation 2
Inoculation 3
HM
NF
GE
inoculates
PHA-blasts
freq. of MBP-specific T cells
f = 5.5 x 10^-7
f = 10^-6
f = 11.6 x 10^-7
Stimulation index
(CPM of PBMC + inoculates or T blasts /control CPM)
Pre . 1 2 4 6 8 . . 58 . 60 . 64 . 68. . 70 . 72 . 76. 80 . .
Pre . 1 2 4 6 8 . . 24 . 26 . 30 . 34 . . 36 . 38 . 42. 46 . .
Pre . 1 2 4 6 8 . . 10 . 12 . 16 . 20 . . 22 . 24 . 28. 32 . .
Time schedule (in weeks)

Figure 4B

MBP-specific
TT-specific
Responding to inoculates
CW
GE
GC
AH
V1
V2
V3
f = 2.3 x 10-6
f = 5.2 x 10-6
f = 0
f = 0
Frequency of specific T cells x 10-7
Time schedule (in weeks)

Figure 5

A
CPM incorporated x10³
150
120
90
60
30
0
No stimulator
Stimulator
TT-specific
CW1G9 CW2F3 GE1B3 GE1D6
as responders
B
% inhibition
100
80
60
40
20
0
autol. inoculates
autol. TT-specific
CW1G9 CW2F3 GE1B3 GE1D6
as inhibitors
C
% specific cytolysis
100
80
60
40
20
0
inoculates
TT-specific
CW1G9 CW2F3 GE1B3 GE1D6
as effectors
D
% specific cytolysis
80
60
40
20
0
No antibody added
+ W6/32
+ HB55
GE1B3.2 GE1D6.5 GE1B8.5 AHF4.2
as effectors

Figure 6

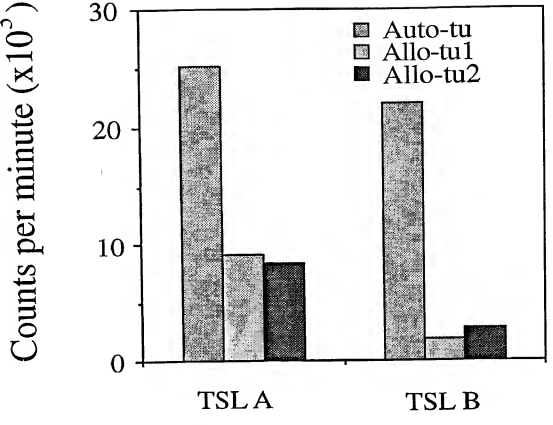
Counts per minute (x10^3)
30
20
10
0
Auto-tu
Allo-tu1
Allo-tu2
TSL A
TSL B

Figure 7

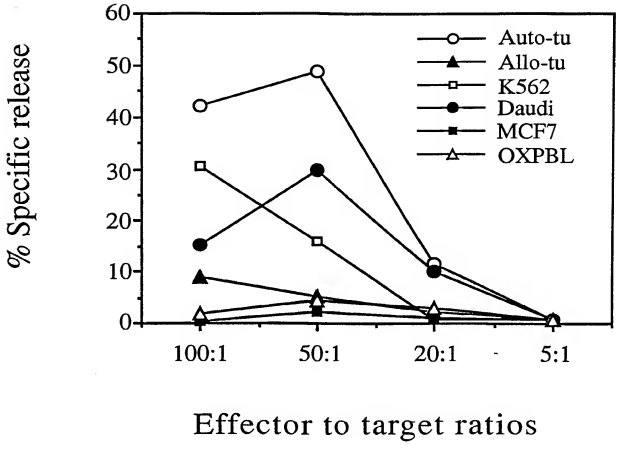
% Specific release
60
50
40
30
20
10
0
Auto-tu
Allo-tu
K562
Daudi
MCF7
OXPBL
100:1
50:1
20:1
5:1
Effector to target ratios

Figure 8

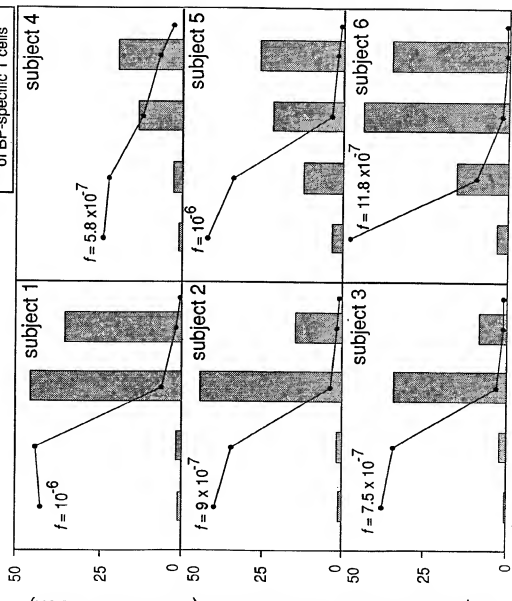
Response to the vaccine clones
Estimated frequency of BP-specific T cells
subject 1
subject 2
subject 3
subject 4
subject 5
subject 6
f = 10^-6
f = 9 x 10^-7
f = 7.5 x 10^-7
f = 5.8 x10^-7
f = 10^-6
f = 11.8 x10^-7
Responses to the vaccine clones (stimulation index)
50
25
0
before
1
2
3
after vaccination

Figure 9

Target TCR
α chain
β chain
V
J
C
V
D
J
C
CDR1
CDR2
other variable regions
CDR3
junctional region
conserved region
germ-line encoded sequence
in the context of MHC
anti-clonotypic T cells
tolerant to the immune system
recognition of a " cross-reactive" clonotypic epitope shared by the immunizing clone and other T cells
recognition of a "private" clonotypic epitope of the immunizing T clone

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 94/00742

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 362 755 (ASAHI KASEI KOGYO KABUSHIKI KAISHA) 11 April 1990 see page 5, line 10 - line 30	1,10,16, 19,42,43
X	EP,A,0 203 403 (ASAHI KASEI KOGYO KABUSHIKI KAISHA) 3 December 1986 see page 11, line 11 - page 13, line 9	1,5, 16-19,25
X	WO,A,85 03948 (CELLTECH LIMITED) 12 September 1985 see page 6 - page 8	1,4,10, 16-18, 42,43
X	WO,A,90 11294 (THE IMMUNE RESPONSE CORPORATION) 4 October 1990 see page 1, line 1 - page 19, line 20 see page 27, line 28 - page 31, line 1	1-25,42, 43

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 1994	14 -06- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	De Kok, A

Form PCT/ISA/210 (second sheet) (July 1992)

International application No.
PCT/EP 94/00742

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 21367 (A.A.VANDENBARK) 10 December 1992 see page 55 - page 84	1-4,6-9, 16-18
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.88, March 1991, WASHINGTON US pages 2466 - 2470 A.BEN-NUN ET AL. cited in the application see the whole document	1-8, 16-18, 31-34
X	NATURE., vol.346, 12 July 1990, LONDON GB pages 183 - 187 K.OTA ET AL. cited in the application see the whole document	1,6-9, 31-34
X	ANNALS OF NEUROLOGY, vol.32, no.3, 1992, BOSTON US pages 330 - 338 J. ZHANG ET AL. cited in the application see the whole document	1-4,6-9, 16-19, 31-34

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/ EP 94/ 00742

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 26-29, 39-41 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged affects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/EP 94/00742

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0362755	11-04-90	JP-A-	2097378	09-04-90
EP-A-0203403	03-12-86	JP-A-	61254527	12-11-86
		JP-A-	62010016	19-01-87
		JP-A-	62012718	21-01-87
		JP-A-	62032879	12-02-87
		DE-A-	3687014	03-12-92
WO-A-8503948	12-09-85	EP-A-	0175705	02-04-86
		JP-T-	61501563	31-07-86
WO-A-9011294	04-10-90	AU-B-	648753	05-05-94
		AU-A-	5356790	22-10-90
		DE-D-	69006018	24-02-94
		DE-T-	69006018	11-05-94
		EP-A-	0463101	02-01-92
		JP-T-	4506512	12-11-92
WO-A-9221367	10-12-92	AU-A-	2147292	08-01-93
		CA-A-	2110055	10-12-92
		EP-A-	0587735	23-03-94

Isolation and characterization of autoreactive proteolipid protein-peptide specific T-cell clones from multiple sclerosis patients

J. Correale, MD; M. McMillan, PhD; K. McCarthy, RN; T. Le, BS; and L.P. Weiner, MD

Article abstract—During the course of multiple sclerosis (MS), myelin proteins are likely antigenic targets for auto reactive T cells. Although most studies have implicated myelin basic protein as a potent encephalitogenic myelin com ponent, proteolipid protein (PLP) appears also to be a possible target antigen in the autoimmune response in MS. I this report, we investigated the human T-cell responses to PLP by using PLP104-117 and PLP142-153 synthetic pep tides as target antigens in limiting dilution. One hundred twenty-five $CD4^+$, T-cell receptor (TCR) $\alpha\beta^+$ T-cell clone (TCCs) were established from the peripheral blood of seven MS patients and five control subjects. Despite the use o enriched cultures no $\gamma\delta$ TCCs were obtained. Recognition of both PLP epitopes occurred in the context of multip HLA-DR alleles. We found no differences in restriction element usage between MS patients and control subjects. Th variable β-region (Vβ) usage was assessed by flow cytometry using a panel of monoclonal antibodies defining differen Vβ elements. In both MS patients and control subjects, there was a marked heterogeneity in the TCR Vβ repertoire Furthermore, sequential evaluation of MS patients during acute attacks and clinical remissions showed even mor broadening of the TCR Vβ repertoire. These data demonstrate that a heterogeneous T-cell response to PLP concerning HLA restriction and TCR usage is present in both MS patients and normal subjects.

NEUROLOGY 1995;45:1370-137

Although the etiology of multiple sclerosis (MS) remains unknown, a large body of evidence[1] supports the hypothesis that autoimmunity plays a significant role in the development of the disease. Further support for this concept is based on studies of experimental allergic encephalomyelitis (EAE),[2] an animal model with clinical and pathologic similarities to MS. In both EAE and MS, activated T cells recognize components of the myelin sheath as immunologically relevant target antigens.[1] Myelin basic protein (MBP) and proteolipid protein (PLP) are the two major constituents of the CNS myelin.[3] MBP is thought to be the putative main target myelin antigen in EAE in many species.[4-6] However, certain strains of mice are susceptible to EAE induced by whole spinal-cord homogenate,[7,8] while being resistant to MBP-induced disease.

In MS patients and healthy controls, MBP-specific T-cell lines and clones can be derived from the peripheral blood. In both groups, T cells proliferate in response to multiple regions of the MBP molecule,[9-11] and respond in a similar manner[12] during MBP-specific cytotoxic assays. Explanations for these data are twofold: (1) MBP-specific T cells may be necessary but not sufficient for development of the disease; (2) in addition to MBP, other components of the myelin membrane may have encephalitogenic capacity.

There is evidence implicating PLP as another myelin component that is encephalitogenic. Investigators have induced acute and chronic EAE by active challenge with either highly purified PLP or peptides based on the sequence of PLP in rabbits,[1] guinea pigs,[14] rats,[15] and mice.[16-20] In addition, adoptive transfer of PLP-sensitized lymph-node cells, PLP-specific T-cell lines, or T-cell clones (TCCs) induces inflammatory demyelination in the CNS of naive recipients.[21-23] Finally, in *SJL/J* mice a strain susceptible to EAE, tolerance can be induced in spinal cord-immunized mice using splenocytes coupled with PLP; this results in a remarkable inhibition of the disease. In contrast, disease

From the Departments of Neurology (Drs. Correale, McMillan, and Weiner, and K. McCarthy and T. Le) and Microbiology (Drs. McMillan and Weiner University of Southern California School of Medicine, Los Angeles, CA.

Supported by the National Multiple Sclerosis Society grants FA 1000-A-1 (JC) and RG 2534-A-1 (JC), and the Norris Foundation (LPW).

Presented in part at the 118th annual meeting of the American Neurological Association, Boston, MA, October 1993.

Received August 30, 1994. Accepted in final form December 2, 1994.

Address correspondence and reprint requests to Dr. J. Correale, Department of Neurology, MCN 142, USC School of Medicine, 1333 San Pablo Stre Los Angeles, CA 90033

duced by spinal cord homogenates cannot be pre-
ted by MBP-coupled splenocytes.[24] Thus, induc-
on of and tolerance to EAE can be specifically and
nificantly modulated by PLP.

While central and peripheral myelin share many
operties, they differ structurally. In the peripheral
ervous system PLP is not present in the myelin
heath,[25] where its function is thought to be per-
ormed instead by P0 proteins.[26] Clinical manifesta-
ions and pathologic studies of MS suggest that the
isease is strictly confined to the CNS.[27] Even
hough there are occasional reports of peripheral
ervous system involvement,[28,29] in ordinary cases,
he evidence is too slight to establish that peripheral
ervous system myelin is attacked during the course
of the disease.[28] Thus, the restriction of PLP to CNS
myelin and the predominantly CNS-limited symp-
oms in MS suggest that PLP would be an appropri-
te target for the autoimmune response.

Whether an autoimmune response directed to
PLP occurs in patients with MS has been a subject
of substantial controversy. Johnson et al[30] provide
evidence for a lack of sensitization to PLP in pe-
ripheral blood lymphocytes among MS patients.
Similarly, TCCs derived from blood, CSF, and MS
plaque tissue did not demonstrate reactivity to
PLP.[31] However, recent data suggest that autoim-
mune recognition of PLP could play an important
role in disease etiology.[32,33]

One requirement to better understanding of the
immune mechanisms in MS is to generate T-cell
lines and clones with specificities against putative
myelin antigens. A limitation in these studies of
human autoreactive T cells is that both naive and
pre-existing autoreactive T cells are stimulated
during culture. Nevertheless, this approach allows
investigation of the potential repertoire of myelin-
activated T cells and represents an important tool
to evaluate the interaction between T-cell receptor
(TCR), major histocompatibility complex (MHC),
and putative myelin antigens.

In this communication we report experiments
designed to characterize the anti-PLP T-cell reper-
toire in MS patients at different stages of the dis-
ease. With this question in mind we analyzed MHC
restriction and TCR variable β-region (Vβ) usage to
determine whether the patterns in MS patients dif-
fer from those found in normal blood donors or if
they change with disease status.

Materials and methods. *Patients.* Eight patients with
linically definite MS (seven patients had relapsing-re-
mitting MS and one patient had chronic progressive MS)
were studied. Patients with a relapsing-remitting course
were studied successively during acute attacks and clini-
cal remissions. None of the patients had received steroids
or immunosuppressant drugs for at least 3 months before
having blood withdrawn. If the patient was in the midst
of an acute exacerbation, blood was obtained before the
administration of IV steroids. Patients re-examined dur-
ing remission were free of steroids for at least 90 days.
Six normal blood donors matched in age, sex, and ethnic
background were studied as a control population. All
subjects were HLA-typed for HLA-A, -B, -C, -DR,
-DRw, and -DQ, using a standard lymphocytotoxicity
assay. Characteristics of the patients and control sub-
jects are summarized in the table. The research project
was reviewed and approved by the USC Institutional Re-
view Board.

Antigens. Native PLP was purified from bovine brain
according to the method of Lees and Sakura[34] and kindly
provided by Dr Stephen Stohlman, Department of Neu-
rology, University of Southern California. Synthetic pep-
tides were synthesized in the Microchemical Core Labo-
ratory at the USC Comprehensive Cancer Center with
an Applied Byosystems model 430 A automated peptide
synthesizer using FAST-MOC chemistry. The peptides
were cleaved from the resin using trifluoroacetic acid,
chromatographed on Sephadex G-10 with 30% acetic
acid, and lyophilized. Each peptide was analyzed by
high-pressure liquid chromatography and was found to
have the expected amino acid composition. Two peptides,
corresponding to fragments 104-117 (KTTICGKGLSA-
TVT) and 142-153 (GKWLGHPDKFVG) of the human
PLP sequence, were synthesized. Selection of the pep-
tides was based on: (1) PLP104-117 peptide overlaps an
encephalitogenic fragment in SWR mice[16] and PLP142-
153 peptide in SJL mice,[17] and (2) both contain amphi-
pathic structures that highly correlate with antigenic
sites on helper T cells.[35]

Tetanus toxoid, *Candida albicans* (both from Accurate
Chemicals, Westbury, NY), and streptolysin O (Sigma
Chemical Co, St. Louis, MO) were used as control antigens.

Generation of PLP-specific T-cell lines and clones. Pe-
ripheral blood mononuclear cells (PBMNC) were isolated
from heparinized peripheral blood by Ficoll/Hypaque
(Pharmacia LKB, Uppsala, Sweden) density gradient.
After isolation, cells were cryopreserved in RPMI 1640
(JRH Biosciences, Lenexa, KS) supplemented with 50%
heat-inactivated fetal calf serum (Gemini Bioproducts
Inc, Calabasas, CA) and 10% dimethylsulfoxide (Sigma
Chemical Co) or used immediately in cultures. For the
establishment of PLP-specific T-cell lines, PBMNC were
resuspended in complete culture medium (RPMI 1640
containing 2 mM L-glutamine, 100 U/ml penicillin, 100
μg/ml streptomycin, all from JRH Biosciences) and 5%
heat-inactivated autologous serum to a final concentra-
tion of 1×10^6 cells/ml. Five million cells were seeded in
25-cm² flasks (Falcon, Becton Dickinson Labware, Lin-
coln Park, NJ) and stimulated with 10 to 25 μg/ml of ei-
ther PLP104-117 or PLP142-153 synthetic peptides.
After incubation at 37 °C in a 5% CO_2 atmosphere for 5
to 7 days, cells were recultured in fresh medium contain-
ing 50 U/ml of recombinant human-IL-2 (r-h-IL-2; a gen-
erous gift from Cetus Co, Emeryville, CA) for an addi-
tional week. Cycles of restimulation with autologous irra-
diated PBMNC (3,000 rad) as antigen presenting cells
(APC) plus antigen, and expansion with r-h-IL-2-rich
medium, were repeated weekly. T-cell lines were restim-
ulated until the response to PLP peptides detected in
proliferation assays (see below) exceeded the response to
control antigens threefold. At this time, usually after
four cycles of restimulation and expansion, cultures were
enriched for PLP-responsive T cells. Clones were derived
by limiting dilution in 96-well round-bottom microtiter
plates (Falcon, Becton Dickinson Labware) at 1 and 0.3
cells/well. Growth in individual wells was identified
using an inverted microscope, and contents of the wells
transferred into 96-well flat-bottom microtiter plates
(Falcon, Becton Dickinson Labware) and restimulated
with PLP peptides in the presence of irradiated autolo-

Table. Clinical characteristics, MHC haplotypes, and number of PLP-peptide–specific T-cell clones isolated per individual from MS patients and healthy donors

Patient	Sex	Age	Clinical picture	Disease duration	Disability (EDSS)	MHC class II*	Number of PLP 104-117 TCCs	Number of P 142-153 TCC
MS patients								
PK	Male	58	Chronic progressive	6 years	4.5	DR15;DRw-;DQ1	11	0
SS	Female	44	Acute attack	5 years	2.0	DR15,17;DRw52;DQ2,6	5	0
			Remission				7	0
RSP	Female	42	Acute attack 1	7 years	3.5	DR1,11;DRw52;DQ1,3	5	0
			Remission				4	3
			Acute attack 2				3	3
RK	Female	43	Acute attack	19 years	3.0	DR9,12;DRw52,53;DQ3	4	3
			Remission				5	4
BD	Male	26	Acute attack	2 years	2.0	DR4,8;DRw52,53;DQ3,4	6	0
			Remission				4	0
BP	Female	32	Acute attack	1 month	2.5	DR12,18;DRw52;DQ3,4	0	4
			Remission				0	4
LW	Female	26	Acute attack	6 months	3.0	DR13,17;DRw52;DQ2	5	4
			Remission				5	3
NR	Female	39	Acute attack	4 years	2.5	DR1,13;DRw52;DQ1,3	0	0
			Remission				0	0
Healthy donors								
JC	Male	33	Control	—	--	DR15;DRw-;DQ6	3	0
KM	Female	39	Control	—	—	DR4;DRw53;DQ8	4	4
BJ	Female	45	Control	—	—	DR1,15;DRw-;DQ1	5	4
RP	Female	28	Control	—	—	DR11,17;DRw52;DQ2,3	6	0
NS	Female	44	Control	—	—	DR7,8;DRw52,53;DQ4,9	5	0
BR	Female	34	Control	—	—	DR4,13;DRw52;DQ1,3	0	0

MHC Major histocompatibility complex.
EDSS Expanded Disability Status Scale.
PLP Proteolipid protein.
TCCs T-cell clones.
* HLA-DR 15 is a split of HLA-DR 2; HLA-DR 17 and HLA-DR 18 are splits of HLA-DR 3; HLA-DR 13 is a split of HLA-DR 6; HLA-DR and HLA-DR 12 are splits of HLA-DR 5.

geous cells. After 3 to 5 days, IL-2-containing medium was added. Cells from individual wells showing a stimulation index (SI) >3 in the proliferation assay were transfered into 24-well plates (Falcon, Becton Dickinson Labware) and expanded. Cloning efficiency was 5 to 9%. Clones were maintained in vitro in medium containing r-h-IL-2 with alternate stimulation every 10 to 14 days as previously described.

Antigen-specific proliferation. TCCs were tested 10 days after the last addition of antigen and feeder cells. Antigen-specific proliferation was examined in a 60-hour assay measuring ^{3}H-thymidine incorporation. Ten thousand T cells and 10^5 adherent, irradiated, autologous PBMNC as APC were cocultured in media alone or in media containing either an optimal concentration of PLP synthetic peptides (10 to 25 μg/ml) or control antigen. For each subject, optimal control antigen and its concentration were determined in preliminary experiments, and were defined as the maximum proliferative response obtained from uncultured PBMNC after stimulation with tetanus toxoid, *Candida albicans*, and streptolysin O, tested over a wide range of antigen concentrations. Twelve hours before harvesting, 1 μCi of ^{3}H-thymidine (ICN Biomedicals Inc, Irvine, CA) was added to each well. Cells were harvested on glass fiber filters (Whatman, Maidstone, England) by an automated cell harvester (Cambridge Technology Inc, Cambridge, MA). ^{3}H-thymidine incorporation was measured in a scintillation counter (Pharmacia LKB Biotechnology Inc, Gaithersburg, MD). The counts per minute thymidine incorporation were calculated as the mean value of triplicate tures.

MHC class II restriction analysis. MHC class II striction elements were identified by monoclonal antibies (mAbs)-blocking experiments. mAbs specific for H DR (Clone L-243), HLA-DQ (Clone SK10), and HLA (Clone B7/21), all purchased from Becton Dickinson, Jose, CA, were added at a final concentration of 1 μg at the onset of the antigen-specific proliferation ass mAb toxic effects were excluded by testing the prolif tive response of TCCs during stimulation with the pl bol ester 12-O-tetradecanoyl-phorbol-13-acetate (T and the calcium ionophore Ionomycin (both from Sig Chemical Co) in the presence of mAbs. In previous exp iments, nonspecific inhibition was excluded by using type-matched control mAbs. Additionally, TCCs were amined by their proliferative responses to PLP pepti using PBMNC sharing only one of the HLA-DR hap types as APCs.

Flow cytometry. Monoclonal antibodies. The mur mAbs fluorescein-isothiocyanate (FITC)-conjugated a Leu-4 (CD3), anti Leu-3a (CD4), anti Leu-2a (CD8), a anti TCRαβ (clone WT31) were purchased from Bec Dickinson. mAbs to human TCR Vβ regions and hum TCRγδ were obtained from T Cell Sciences (Cambrid MA), including mAbs recognizing Vβ5.1 (clone LC Vβ5.2 and 5.3 (clone 1C1); Vβ5.3 (clone W112); V (clone OT145); Vβ8 (clone 16G8); Vβ12 (clone S51 Vβ13 (clone BAM13); Vβ3 (clone 8F10); and the 5 ch of the human γδ TCR. TCR5.1 (clone 5A6.E9), mAbs

tained from other sources included: anti Vβ1 (clone AHUT71 and anti Vβ9 (clone AMKB1-2), both purchased from Pharmingen (San Diego, CA); and anti Vβ2 (clone E22E7.2), anti Vβ17 (clone BA-62), and anti Vβ19 (clone E17.5F3.15.13), purchased from AMAC Inc (Westbrook, ME). FITC-labelled goat anti-mouse IgG F(ab)$_2$ (Jackson Immunoresearch Laboratories, West Grove, PA) was used as a secondary reagent for visualization of unlabeled antibodies.

Immunofluorescence analysis. Cloned T cells (10^4 cells/sample) were resuspended in 50 μl of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide (both from Sigma Chemical Co). For direct staining, optimal concentrations (100 to 400 ng) of FITC-conjugated mAbs were added and the samples incubated for 45 minutes at 4 °C. For indirect staining, after incubation with the primary mAb, cells were washed in PBS/BSA, and FITC-goat F(ab)$_2$ anti-mouse IgG antibody was applied as second-step reagent. Cells were further incubated for an additional 30 minutes on ice. Each sample was washed once or twice in PBS/BSA and analyzed by flow cytometry using a FACSTAR cell sorter (Becton Dickinson and Co, Mountain View, CA). Nonspecific binding was excluded using isotype-matched, labeled control mAbs.

Results. *Generation of autoreactive PLP TCCs.* PBMNC from eight MS patients and six healthy blood donors were stimulated with synthetic PLP peptides (human sequences 104-117 and 142-153) and further expanded in medium containing r-h-IL-2 at weekly intervals. Proliferating T cells subsequently were cloned by limiting dilutions. T-cell lines and clones were successfully generated from seven of eight MS patients and five of six control subjects. A total of 94 clones was derived from MS patients and 31 clones from control individuals. Sixty-six of the 94 MS TCCs responded to the fragment PLP104-117 (SI: 3 to 39) and 28 MS TCCs showed reactivity against the fragment PLP142-153 (SI: 4.5 to 144). Twenty-three TCCs derived from healthy controls were specific for PLP104-117 (SI: 5.3 to 128) and eight TCCs for PLP142-153 (SI: 11 to 85). The number and specificity of TCCs isolated from each donor are provided in the table. The reactivity patterns of the TCCs were specific, since they failed to proliferate in response to control antigens and nonstimulatory PLP fragments. Figure 1 shows the proliferative response of representative TCCs derived from controls JC and BJ and patients SS and LW.

To analyze the T-cell repertoire during acute attack and after clinical recovery, MS TCCs from six patients with relapsing-remitting disease were established from PBMNC obtained during acute attack and 3 months later during clinical remission (table). TCCs obtained from patient RSP during a first sampling (acute attack 1) showed specificity only for peptide PLP104-117. TCCs generated 3 and 7 months later during remission and a second acute attack showed a broader specificity, responding to both PLP104-117 and PLP142-153. Note that TCCs isolated during acute attack 1 and TCCs derived from patient RK were generated using similar culture conditions in parallel experiments. TCCs isolated from patient RK showed reactivity against both PLP peptides. This fact argues against the possibility that a lack of reactivity to PLP142-153 during the first sampling represents an artifact of the T-cell cloning procedure. Taken together, these data indicate that, after successive attacks, some MS patients may display new PLP T-cell specificities, and, as a consequence, a significant epitope heterogeneity is generated.

The cell-surface phenotype of all the clones tested was CD3$^+$ CD4$^+$ CD8$^-$ TCRαβ$^+$. Since little is known about antigen specificity of γδ T cells during the course of MS one goal was to isolate and characterize PLP γδ autoreactive TCCs. Because γδ T cells represent a small fraction of CD3$^+$ T cells (3 to 5% in normal blood donors), TCR γδ$^+$ cells were initially sorted from PBMNC of five patients with PLP-responsive MS (SS, RSP, RK, LW, and BD) and were propagated as described in "Materials and methods." Despite the use of enriched cultures, γδ T cells did not respond to any of the PLP peptides examined, suggesting that either γδ T cells recognize different autoantigens or PLP epitopes, or they are not truly autoreactive T cells, but bystander cells in MS lesions.

MHC restriction. Thirty-nine TCCs derived from six MS patients and 10 TCCs generated from three healthy donors were studied for their HLA restriction. The HLA class II molecules that served as restriction elements for PLP-specific TCCs were determined by inhibition of T-cell proliferation by anti-DR, anti-DQ, and anti-DP mAbs and were further analyzed in proliferation assays using semi-allogenic PBMNC as APCs. All the TCCs were restricted by HLA-DR gene products as judged from inhibition by HLA-DR mAbs. In each case antigen-specific proliferation was inhibited among 78 to 95%. None of the TCCs studied was inhibited by mAbs specific for HLA-DQ or HLA-DP. Representative results obtained with TCCs from patients SS, LW, and RSP and healthy donor BJ are shown in figure 2.

Despite the differences in peptide specificity, the T-cell proliferative responses were blocked equally well by anti-HLA-DR mAb in both PLP104-117 TCCs and PLP142-153 TCCs. Thus, the observed HLA-DR restriction seems to be independent of the peptide specificity of the TCCs. TCCs were further characterized by using PBMNC sharing only one of the HLA-DR alleles. Five different DR alleles were identified as restriction elements for PLP104-117 (DR1, DR4, DR9, DR13, and DR15) and six different DR alleles restricted PLP142-153 presentation (DR1, DR4, DR9, DR13, DR15, and DR18). In control individual BJ, PLP142-153 was recognized in the context of two different DR alleles: DR1 and DR15. Interestingly, even though both patient NR and control subject BR expressed HLA-DR antigens linked to MS susceptibility (HLA-DR4 and HLA-DR13), they did not show reactivity to any of the PLP epitopes. Differences in restriction ele-

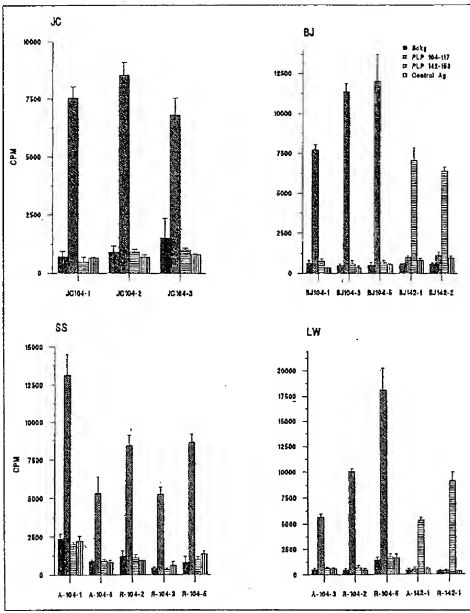

Figure 1. Antigen-induced proliferation of autoreactive proteolipid protein (PLP) T-cell clones derived from patients SS and LW and healthy controls JC and BJ. The proliferative response of each clone to PLP peptides was determined by coculture of 10^4 T cells with 10^5 adherent irradiated peripheral blood mononuclear cells and medium alone (Bckg); control antigen (Ag) (streptolysin O); stimulatory PLP peptide; or non-stimulatory PLP fragment for 60 hours. Proliferation was determined by ^{3}H-thymidine incorporation. Results are shown as mean value of counts per minute (CPM) ± SE for triplicate cultures.

ment usage between MS patients and healthy blood donors were not found.

TCR usage. PLP-specific TCCs were analyzed for their TCR Vβ regions usage by flow cytometry using a panel of 13 mAbs defining 11 of the 24 Vβ families identified so far. A similar panel of mAbs was recently used in other studies to characterize the human TCR repertoire in normal and pathologic conditions.[36,37] Forty-eight PLP-TCCs (51%) from MS patients and 15 TCCs (48%) from healthy blood donors were stained by one of the mAbs used. In the MS patients, a marked heterogeneity in the TCR Vβ repertoire was observed within and between patients. Diversity was found even among TCCs that recognized the same epitope in association with the same MHC class II restriction element. Only TCCs from patient PK (a patient with chronic progressive MS) showed a trend to use preferentially TCR Vβ 5.1 (5 of 11 clones). Nevertheless, they used a minimum of four different TCR Vβ elements for recognition of PLP104-117. The remaining TCCs expressed Vβ2 and Vβ17; four TCC expressed unknown Vβ regions. Furthermore, TCCs generated during acute attack(s) and remission in the same patient and in response to the same epitope showed a wide diversity of TCR Vβ elements, broadening even more the TCR Vβ repertoire. A similar extent of heterogeneity of TCR Vβ usage was observed in TCCs derived from control individuals.

Discussion. A major question in MS is the nature of the antigen(s) against which the immune response is directed. Although an autoimmune re-

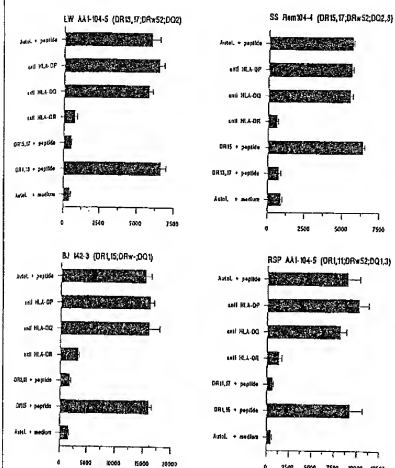

Figure 2. HLA-D.R restriction of autoreactive proteolipid protein (PLP) T-cell clones derived from patients SS, LW, and RSP, and healthy control BJ. T-cell clones (10^4/well) were cultured with PLP peptides at a final concentration of 10 to 25 μg/ml in the presence of adherent irradiated autologous peripheral blood mononuclear cells (PBMNC) as antigen presenting cells (APC) (10^5/well) for 60 hours. Monoclonal antibodies specific for HLA-DR, HLA-DQ, and HLA-DP were added to the cultures at a final concentration of 1 μg/ml at the onset of the proliferation. In addition, PBMNC sharing only one HLA-DR allele were used as APC to analyze further the specific HLA-DR restriction. Proliferation is represented as the mean value of counts per minute (CPM) ± SE for triplicate cultures.

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sponse arises against the intact PLP molecule and different PLP peptides in MS,[32,33] little is known about the PLP T-cell repertoire in MS patients and normal subjects. Furthermore, few studies have examined the T-cell repertoire in MS patients over time. In this study we investigated the relevance of PLP as a putative target antigen for autoimmune attack by selecting TCCs from MS patients during acute attacks and clinical remissions and from normal controls, using two different PLP synthetic peptides as target antigens. To our knowledge, this is the first study on the TCR Vβ usage in autoreactive PLP T-cells during the course of MS.

The PLP fragments used in this study, PLP104-117 and PLP142-153, overlap encephalitogenic sequences in SWR/J[16] and SJL/J[17] mice. The amino acid sequences of mouse and human PLP are identical.[18] While our studies were in progress, different laboratories reported additional PLP fragments that induce EAE in different strains of mice.[19-29] In MS patients, previous studies have indicated that PBMNC can recognize PLP peptides 88-108, 103-116, and 139-154 in proliferation assays.[23] In addition, Pelfrey et al[29,40] established and characterized a group of T-cell lines derived from the peripheral blood of MS patients using PLP40-60[39] and PLP89-106[40] as the target antigens. Thus, a variety of PLP peptides can be recognized by T cells from MS patients and normal controls, emphasizing the complexity of the immune response to PLP in humans.

As MS develops, sensitization to new myelin antigens might occur. In one MS patient (RSP), studied longitudinally during two consecutive attacks and remission, we observed a diversification of the autoreactive T-cell repertoire due to broader peptide recognition. We used only two PLP epitopes during this study, and most probably some encephalitogenic epitopes as well as cryptic sequences were not represented in our investigation. Therefore we could not completely evaluate the immune response to new antigenic determinants arising during the course of MS. Several lines point to the idea that because of the myelin breakdown in chronic relapsing EAE, T cells can recognize cryptic MBP epitopes,[41] resulting in a subsequent sensitization to other myelin proteins.[42,43] Furthermore, most of the MS patients who showed recognition of multiple PLP peptides also showed significant reac-

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tivity against MBP,[32] supporting the possibility of a progressive broadening of the autoimmune response after successive attacks. Interestingly, patient PK, who had had chronic progressive disease for 6 years, showed reactivity to only one peptide. This observation suggests that the patient's autoimmune repertoire evolves during the course of the disease, but our investigation probably did not evaluate the determinants that contribute to disease progression, as previously discussed. Alternatively, in accordance with previous observations,[44,45] a chronic antigenic stimulation during the course of MS may lead to a clonal expansion followed by clonal exhaustion with the consequent restriction of T-cell reactivity.[46]

Studies identify T cells bearing the γδ TCR colocalizing with heat shock protein (hsp) in MS plaques.[47,48] The limited genetic diversity of these infiltrating T cells indicates that a clonal expansion selected by a specific antigen occurs in the CNS.[48] This is clearly different from the peripheral blood, where MS patients do not show γδ reactivity to myelin antigens[49] (our observations). Similarly, a selective expansion of γδ-bearing T cells occurs in the synovial membrane of patients with rheumatoid arthritis.[50] Thus, if γδ T cells are truly involved in the initiation of the demyelinating process, they could recognize in the CNS an antigen different from MBP or PLP (ie, hsp).[47,48] A second possibility is that γδ T cells arise within the MS lesions by a secondary recruitment after the inflammatory process has begun, thus contributing to chronicity of the lesions.[51]

Our analysis of HLA restriction elements demonstrates that the proliferative responses of PLP-specific TCCs were exclusively restricted by HLA-DR. In both patients and normal controls, recognition of epitopes 104-117 and 142-153 occurs in the context of multiple HLA-DR alleles. Other antigens derived from infectious agents[52] or autoantigens, particularly MBP,[53-55] exhibit promiscuous binding of peptides (having the capacity of binding to different HLA-DR alleles). A promiscuous binding does not contradict the idea that the MHC plays an important role in the selection of the T-cell repertoire, since a single peptide can bind with different affinities to several HLA-DR alleles.[56] Variations in autoantigen binding affinity to MHC class II molecules appear to have an important effect on determining two divergent responses, either immunodominance during an autoimmune response[56] or T-cell tolerance during thymic selection.[57] Thus, even though the presence of some MHC class II susceptibility molecules (HLA-DR2, DR4, DR6) seems to play an important role in permissiveness to develop an autoimmune response, during the course of MS a hierarchical and competitive binding of peptides by MHC class II molecules represents another important element in determining disease susceptibility.[56]

A marked restriction in the TCR Vβ gene repertoire has been demonstrated in MBP-specific TCCs derived from B10 PL and PL/J mice[58,59] and th Lewis rat[60] during the course of EAE, suggesting critical role for those TCRs in the pathogenesis EAE, and providing a molecular target for possibl specific treatments.[61] In humans, however, TCI Vβ repertoire studies using MBP-specific TCC have yielded conflicting results.[51,62-66] Unlike MBP the TCR Vβ repertoire of PLP-specific T cells i MS patients has not been studied. Our data show a marked heterogeneity of TCR Vβ usage, withi and between patients, among TCCs specific fo PLP104-117 and 142-153. Furthermore, we observed a broader TCR Vβ repertoire in patient studied successively during acute attacks and clinical remissions. Our results confirm previous observations that TCCs that recognize the same peptide sequence in the context of similar HLA-DR restriction express a diverse set of TCR Vβ regions.[54,62] Although the TCR Vβ mAbs used in this study cover only part of the total T-cell population approximately 50% of the TCCs generated were stained by one of these mAbs, and in most of the MS patients and control subjects we identified at least three to four different Vβ segments. Of particular interest is the fact that, in contrast to the MBP immune response, the TCR Vβ repertoire among PLP-specific TCCs derived from SJL/J mice with EAE has been shown to be markedly heterogeneous.[67]

In summary, we show that autoreactive PLP T cells are part of the T-cell repertoire in both MS patients and normal subjects. In both groups we identified different levels of diversity during the course of the disease, ie, recognition of different epitopes promiscuous HLA-DR restriction, and heterogeneous TCR repertoire. Therefore, the design of immunotherapies based on the use of specific peptide targeting restricted T-cell populations may be more complex than originally anticipated.

Note. Readers can obtain five pages of additional information from th National Auxiliary Publications Service, c/o Microfiche Publications, PO Box 3513, Grand Central Station, New York, NY 10163-3513. Request document no. 05179. Remit with your order (not under separate cover) US funds only, $7.75 for photocopies or $4.00 for microfiche. Outside the United States and Canada, add postage of $4.50 for the first 20 pages and $1.00 for each 10 pages of material thereafter, or $1.75 for the first microfiche and $.50 for each fiche thereafter. *There is a $15.00 invoice charge on all orders filled before payment.*

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Anti-Myelin Basic Protein and Anti-Proteolipid Protein Specific Forms of Multiple Sclerosis

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Human myelin basic protein (hMBP) and proteolipid protein (PLP) were used as antigens in a solid-phase radioimmunoassay to determine relative frequencies of anti-MBP and anti-PLP in cerebrospinal fluid (CSF) of optic neuritis and multiple sclerosis (MS) patients. Forty-nine of 55 patients with optic neuritis had increased CSF anti-MBP and the remaining 6 had increased anti-PLP. Of 385 MS patients, MS relapse: 173 of 180 patients had increased anti-MBP, 5 of the remaining 7 patients had elevated anti-PLP, and 2 had neither of these autoantibodies. Progressive MS: 111 of 116 patients had increased anti-MBP in either free and/or bound form, of the remaining 5 patients 4 had increased anti-PLP, and 1 had neither anti-MBP nor anti-PLP. MS remission: 15 of 87 patients had somewhat increased anti-MBP, none had anti-PLP. IgG was purified by affinity chromatography from necropsy central nervous system (CNS) tissue samples of 4 individual patients with clinically definite and neuropathologically confirmed MS. Three of these 4 patients who had increased levels of CSF anti-MBP also had increased anti-MBP titers in CNS tissue-extracted IgG. The fourth patient who had anti-PLP in CSF also had anti-PLP in brain tissue IgG. These autoantibodies were not detected simultaneously in any patient. These results suggest that there are at least two immunologically distinct forms of MS, i.e., a common form highly associated with anti-MBP and more frequent prominent inflammatory characteristics in CSF and CNS, and an infrequent form associated with anti-PLP in CSF and tissue, and less abundant inflammation. Anti-MBP purified from CNS tissue IgG by antigen-specific affinity chromatography was reacted with synthetic peptides of hMBP. The anti-MBP epitope on the hMBP molecule was restricted between residues 75 and 106. The PLP epitope for anti-PLP has not as yet been determined. These observations have theoretical implications for anticipated future specific immunotherapy of MS.

Warren KG, Catz I, Johnson E, Mielke B. Anti-myelin basic protein and anti-proteolipid protein specific forms of multiple sclerosis. Ann Neurol 1994;35:280–289

Multiple sclerosis (MS) is an acquired demyelinating disease of the human central nervous system (CNS) that occurs more commonly in temperate than tropical climates, is more common in occidental rather than oriental races, and is also more common in females than in males [1–4]. Genetic susceptibility or resistance to this disease is thought to be associated with genes within or close to the HLA-DR-DQ subregion located on the short arm of the sixth chromosome [5]. Although epidemiological studies are suggesting that an environmental trigger may be necessary, infectious agents have not been found reliably in association with the diseased brain tissue. The concept of an autoimmune mechanism operational in the demyelination process associated with MS is currently entertained. Circumstantial evidence to support this idea consists of the following three observations: (1) Inflammation is a reliable associated pathological feature, (2) perivenous demyelination/inflammation occurs not only in MS but also in parainfectious encephalomyelitis, and (3) experimental allergic encephalomyelitis (EAE) is an accepted animal model of MS with somewhat similar clinical and pathological features, produced by inoculating a susceptible host with either myelin basic protein (MBP) or proteolipid protein (PLP) or one of their encephalitogenic synthetic peptides in conjunction with Freund's complete adjuvant [6–12].

Because a reliable feature of MS is increased intra blood–brain barrier (BBB) IgG synthesis [13, 14], our previous research has concentrated on potential humoral autoimmune mechanisms of demyelination vis-à-vis myelin proteins. Antibodies to MBP (anti-MBP) are regularly found in cerebrospinal fluid (CSF) of patients with acute optic neuritis and active MS as well as in CNS tissue of MS patients [15–19]. The anti-MBP epitope range on the human MBP molecule has been

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approximated by synthetic peptide studies [18, 20, 21]. Despite the high association of anti-MBP with the majority of MS patients, not every patient was observed to have this autoantibody even when the disease was acutely relapsing or rapidly progressing; such patients devoid of anti-MBP may have one or more autoantibodies directed against other myelin proteins.

The major purpose of this study was to determine relative frequencies of anti-MBP and anti-PLP in CSF of a large population of optic neuritis and MS patients, and to look for these two autoantibodies in MS CNS tissue. Our first hypothesis was that because anti-MBP can be detected in the majority of optic neuritis CSFs [16, 17, 20], anti-PLP would be less frequently or even randomly observed. The second hypothesis was that anti-PLP would also be infrequently detected in MS CSF. Finally, the third hypothesis was that if anti-MBP and/or anti-PLP were detected in CSF, then the homologous autoantibody(ies) will be found in CNS tissue from the same patient.

Methods

Selection of Cerebrospinal Fluid and Multiple Sclerosis Tissue

Between 1978 and 1992, 2,485 patients with optic neuritis and MS were registered with the Northern Alberta Multiple Sclerosis Patient Care and Research Clinic of the University of Alberta in Edmonton, Canada. Optic neuritis patients were usually referred by an ophthalmologist, whereas MS patients were referred by general practitioners from Edmonton and Northern Alberta. The diagnosis of MS was established after clinical review (KGW) and appropriate laboratory testing including evoked responses and magnetic resonance imaging (MRI) as well as CSF analysis. Matched CSF and serum samples were obtained from a total of 440 patients who participated in this study, i.e., 55 patients with optic neuritis and 385 with clinically definite MS [22]. IgG and albumin levels were determined in all CSF and serum samples by standard methods in the Department of Laboratory Medicine, University of Alberta Hospitals; CSF was tested further for oligoclonal banding and levels of anti-MBP and anti-PLP. CNS tissue was obtained from 4 patients with clinically definite and neuropathologically confirmed MS (EJ, BM); a portion of each brain and spinal cord was used for neuropathology confirmation and the remainder was used for antibody studies.

Preparation of Myelin Antigens

Human white matter from non-MS brain was used to simultaneously prepare MBP and PLP. Both proteins were extracted and purified at 4°C to minimize proteolytic cleavage. White matter was homogenized with 19 (× weight) volumes of chloroform/methanol (2:1), mixed overnight (16 ± 1 hr), and filtered through Whatman no. 1 filter paper.

The solid was further used to prepare MBP as previously described by Deibler and associates [23]. Furthermore, MBP was purified by gel filtration on Sephadex G-150 (Superfine, Pharmacia). The purified protein was collected with 0.1 M hydrochloric acid in two to three fractions with A_{280} monitoring. After polyacrylamide gel electrophoresis (Fig 1), only protein fractions that produced a single band of 18.5 kd were pooled, freeze-dried, and stored at −80°C for further use in radioimmunoassays (RIAs).

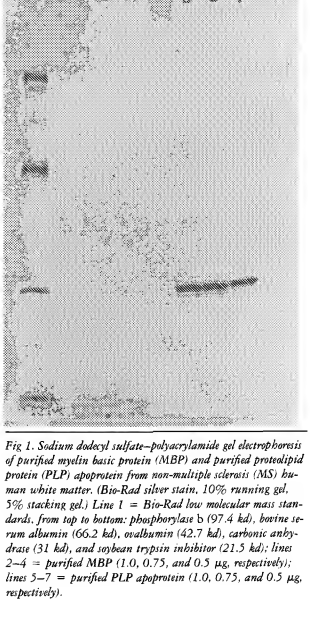


Fig 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified myelin basic protein (MBP) and purified proteolipid protein (PLP) apoprotein from non-multiple sclerosis (MS) human white matter. (Bio-Rad silver stain, 10% running gel, 5% stacking gel.) Line 1 = Bio-Rad low molecular mass standards, from top to bottom: phosphorylase b (97.4 kd), bovine serum albumin (66.2 kd), ovalbumin (42.7 kd), carbonic anhydrate (31 kd), and soybean trypsin inhibitor (21.5 kd); lines 2–4 = purified MBP (1.0, 0.75, and 0.5 μg, respectively); lines 5–7 = purified PLP apoprotein (1.0, 0.75, and 0.5 μg, respectively).

PLP was isolated and further purified from the chloroform/methanol filtrate [24]. After vigorous mixing with 0.2 volumes of distilled deionized water, the filtrate was centrifuged at 1,000 *g* for 30 minutes and the top aqueous layer was discarded. The remaining organic phases (interface + lower phase) were solubilized with methanol and evaporated under a stream of N_2 to two-thirds of the initial volume, and proteins were precipitated with 5 volumes of ether. Tubes were further centrifuged at 1,000 *g* for 30 minutes, the precipitate was dissolved in an eluant consisting of chloroform/methanol (1:1) containing 5% of 0.1 M HCl and applied to a column of Sephadex LH-60 (Pharmacia) [25]. Proteins were col-

lected within void volume (one to two fractions) with A_{280} monitoring and precipitated again with 5 volumes of ether as described above. The precipitated proteins containing mainly PLP and DM-20 were dissolved in a mixture of chloroform/methanol/water (4:4:1) and applied to a column of CM-Sepharose (Pharmacia) for further separation [26]. The column was eluted with 15 ml of chloroform/methanol/water (4:4:1), 20 ml of this mixture containing 0.01 M ammonium acetate, 50 ml containing 0.05 M ammonium acetate, 15 ml containing 0.075 M ammonium acetate, and 50 ml containing 0.10 M ammonium acetate. (A continuous gradient of 200 ml of 0.05 to 0.20 M ammonium acetate can also be used with reasonable results.) DM-20 was collected in the 0.05 M ammonium acetate and PLP came off in 0.10 M ammonium acetate. Appropriate protein fractions were pooled, desalted on Sephadex LH-20 (Pharmacia) with chloroform/methanol (1:1) and 5% of 0.1 M HCl as eluant, converted to water-soluble form [27], and stored frozen at −80°C at a concentration of 1 mg/ml. Purity of PLP was further checked by reverse-phase high-pressure liquid chromatography with a linear gradient of 40% to 100% of 1-propanol in 0.1% trifluoroacetic acid over a 20-minute period with A_{280} monitoring and by polyacrylamide gel electrophoresis (see Fig 1).

Radioimmunoassay for Anti-Myelin Basic Protein and Anti-Proteolipid Protein

Anti-MBP was detected by a solid-phase RIA as previously described [15]. Anti-PLP was also measured by an RIA similar to that for anti-MBP, i.e., Immulon microtiter wells were coated with 100 μl of 10 μg/ml of water-soluble PLP (1 μg/well) and incubated overnight at 37°C. After quenching with bovine serum albumin (BSA) and three water washes, the wells were stored for future use at 4°C. Samples of 100 μl of CSF or tissue extracts diluted to 0.010 gm of IgG/L (with 0.01 M PBS, 0.05% Tween 20) were incubated in PLP-coated wells for 16 to 24 hours at room temperature. After six buffer washes (three with 0.01 M PBS, 0.05% Tween 20, and three with PBS alone), wells were incubated with goat anti-rabbit IgG-Fc specific (in 0.01 M PBS, 0.05% Tween 20, 0.5% BSA) for 2 hours at 37°C and then rinsed as above. Finally, ^{125}I-protein A (or ^{125}I-protein G) was added and incubated for 5 hours at room temperature. When ^{125}I-protein G was used as tracer, ovalbumin replaced BSA in assay buffer and for quenching. After three final PBS washes, the wells were individually counted. Like anti-MBP, assay results are expressed in radioactivity units as follows: (counts of sample − counts of blank) ÷ (counts of total radioactivity − counts of blank). All samples are run in quadruplicate and counting time is 10 minutes in order to collect >10,000 counts for any positive sample. Within-assay reproducibility is between 3% and 5% and between-assay variation in between 4% and 7%. Blanks or nonspecific binding are performed for each sample in uncoated wells. Because our antigen-PLP apoprotein is free of lipids, counts for nonspecific binding are negligible (≤0.5% of total radioactivity). A hyperimmune anti-PLP serum (rabbit) and subsequently a pool of PLP-positive CSFs were used as positive controls (25 ± 1.5 and 15 ± 1.0 radioactivity units, respectively). Results of ≤4 radioactivity units are considered negative for both anti-MBP and anti-PLP.

Purification of IgG from Central Nervous System Tissue

Extracellular (free) as well as tissue-bound IgG was isolated at 4°C from CNS tissue (brain, spinal cord, and optic nerves) obtained from 4 individual MS patients [18, 19]. All tissues were cut into thin slices and extensively washed with normal (0.15 M) saline until there was no more apparent blood (albumin levels in the wash, ≤0.035 gm/L).

FREE (UNBOUND) PROTEIN EXTRACTS. Individual tissue samples from different areas of the CNS (brain, spinal cord, and/or optic nerves) were homogenized with 10 (× weight) volumes of a neutral buffer (0.01 M PBS, pH 7.2) containing 0.2% sodium azide, 0.005 M pepstatin (Sigma), and 0.004 M ε aminocaproic acid, and mixed for 20 minutes. The 10% suspensions were centrifuged for 1 hour at 100,000 *g* in a Beckman L7-35 Ultracentrifuge. Extraction was performed twice in the same manner until the protein concentration in the neutral wash was undetectable. Pooled "free protein extracts" from each individual area of individual patients were assayed for total protein, IgG, and immunoreactive albumin, concentrated five to ten times, checked for anti-MBP and anti-PLP activity, and used to purify IgG by affinity chromatography.

TISSUE-BOUND PROTEIN EXTRACTS. When exhausted of "free" proteins the pellet was homogenized to a 20% suspension with an acid buffer (0.1 M glycine-HCl, pH 2.2, containing 0.05 M ε aminocaproic acid and 0.2% sodium azide), mixed for 1 hour, and then centrifuged at 100,000 *g*. The clear supernatant or "tissue-bound protein extract," was immediately dialyzed to neutral, assayed for total protein and IgG, concentrated five times, checked for anti-MBP and anti-PLP activity, and further used to prepare IgG by affinity chromatography.

PURIFICATION OF IgG BY AFFINITY CHROMATOGRAPHY. IgG was purified from free and tissue-bound protein extracts by affinity chromatography on protein A (or G)-Sepharose 4 Fast Flow (Pharmacia) as previously described [18, 19]; an aliquot of concentrated free or tissue-bound protein extract initially filtered through a 22-μm Milex GS filter (Millipore, Canada) was applied to the affinity column; non-IgG proteins were eluted at neutral pH; IgG was released with 0.1 M glycine-HCl (pH 2.5–2.7). IgG containing fractions (usually two to four) were pooled and immediately dialyzed to neutral. IgG purified from free and bound protein extracts (free IgG and tissue-bound IgG, respectively) migrated as a single band in polyacrylamide electrophoresis under nonreducing conditions and as heavy and light chains in the presence of mercaptoethanol. Free and tissue-bound purified IgG retained anti-MBP or anti-PLP activity detected in corresponding whole protein extracts.

Isolation of Anti-Myelin Basic Protein from Purified IgG

IgG purified from free and bound hydrosoluble protein extracts of MS CNS tissue was used as starting sample to isolate anti-MBP by MBP-specific affinity chromatography on an MBP-Sepharose column [28]. When purified anti-MBP was

Table 1. Cerebrospinal Fluid Data in One Group of Patients with Optic Neuritis and Three Groups of Patients with Multiple Sclerosis

		Intra BBB IgG Synthesis					Anti-MBP				Anti-PLP		
Diagnosis	Number	IgG Index[a]	n ↑	Daily Synthesis[b]	n ↑	OB[c]	Free	n ↑	Bound	n ↑	Free	n ↑
Optic neuritis	55	0.90 ± 0.41	34	10.7 ± 11.2	21	28	9.1 ± 3.1	49	2.7 ± 1.5	7	0.5 ± 3.0	6
Multiple sclerosis	385											
Relapse	180	1.17 ± 0.52	161	11.5 ± 4.0	168	113	9.3 ± 4.5	173	3.8 ± 3.0	30	1.2 ± 1.91	5
Progressive	116	1.32 ± 0.40	104	11.0 ± 3.0	104	62	5.4 ± 3.0	73	12.1 ± 7.7	101	0.9 ± 1.52	4
Remission	89	0.72 ± 0.22	47	5.5 ± 2.5	49	49	2.3 ± 3.1	15	2.9 ± 4.9	15	0	0

Cerebrospinal fluid data including estimates of intra blood–brain barrier (BBB) IgG synthesis and levels of anti-myelin basic protein (anti-MBP) and anti-proteolipid protein (anti-PLP) in a group of patients with optic neuritis and 3 groups with multiple sclerosis. Free anti-MBP is detected before acid hydrolysis of cerebrospinal fluid (CSF). Bound anti-MBP is detected after acid hydrolysis of CSF. Anti-MBP and anti-PLP results expressed in radioactivity units; see Methods for definition and range.

[a]Link's IgG index.
[b]Tourtelotte's empirical formula for daily IgG synthesis.
[c]Oligoclonal banding by polyacrylamide gel isoelectric focusing (pH 3–10). Samples with matching bands in serum due to damaged BBB are read negative.

n ↑ = number of patients with abnormal results.

absorbed with MBP, postabsorption supernatants had undetectable IgG, thus demonstrating the purity of antibody preparations.

Anti-PLP was not further purified from free or tissue-bound IgG.

Synthetic Peptide Specificity of Free and Tissue-bound Anti-Myelin Basic Protein Purified from Multiple Sclerosis Central Nervous System

SYNTHETIC PEPTIDES OF HUMAN MBP. Three different sets (set 1, set 2, and set 3) of progressively more refined synthetic peptides were used to narrow the anti-MBP epitope on the human MBP molecule. All synthetic peptides were prepared by the FMOC (9-fluorenylmethoxycarbonyl) method by Dr Nigel Groome at Oxford Polytechnic and kindly donated for epitope studies [29, 30]. Set 1 used in our previous CSF and tissue studies included 18 synthetic peptides each of different size (8–25 residues), covering most of the length of human MBP [18–21]. Set 2 included 24 different synthetic peptides of equal size (15 residues each), overlapping each other by 7 residues and covering the entire length of human MBP. Set 3 included 11 synthetic peptides of 10 to 25 residues covering the area corresponding to residues 75 to 95 of human MBP.

To determine the epitope range of anti-MBP purified from MS CNS on the human MBP molecule, free and tissue-bound purified antibody were reacted with increasing amounts (100–10,000 ng) of each of the 53 synthetic peptides, initially in a liquid-phase assay, and then anti-MBP levels were determined in all mixtures by a solid-phase RIA as previously described [15, 31].

Results

Cerebrospinal Fluid Data of Optic Neuritis and Multiple Sclerosis Patients

Estimates of intra BBB IgG synthesis as well as levels of anti-MBP and anti-PLP were determined in CSF samples obtained from 55 patients with optic neuritis and 385 clinically definite [22] MS patients (Table 1). Of 55 optic neuritis patients, 34 showed an increased IgG index, 21 had increased daily IgG synthesis, and oligoclonal banding was positive in 28. Free levels of anti-MBP were elevated in 49 of 55 and 7 of these 49 patients had increased levels of bound anti-MBP; 15 of 49 patients with elevated anti-MBP had normal IgG synthesis and negative oligoclonal banding. The 6 remaining patients with undetectable anti-MBP had increased levels of anti-PLP. Both antibodies were not simultaneously detected in any of these 55 optic neuritis CSFs.

Three hundred eighty-five MS patients were clinically divided into 3 groups as follows: acute relapses, progressive, and remission (see Table 1). In a group of 180 patients with acute relapses, 161 had an increased IgG index, 168 had elevated levels of daily IgG synthesis, and 113 illustrated positive CSF oligoclonal banding. Free anti-MBP was elevated in 173 of 180 patients and 30 of 173 had increased bound anti-MBP; 4 of 173 patients with elevated anti-MBP had normal intra BBB IgG synthesis and negative oligoclonal banding. A total of 7 of the 180 acute relapse patients had undetectable CSF anti-MBP; 5 of these 7 patients had increased CSF anti-PLP, whereas in the remaining 2 patients neither anti-MBP nor anti-PLP were detected. In a group of 116 patients with chronic progressive MS, 104 had an increased IgG index, 104 had increased daily IgG synthesis, and 62 had oligoclonal bands in their CSF. Anti-MBP was elevated in 111 of 116 patients; 10 had increased free anti-MBP, 63 had increased free and bound anti-MBP, and 38 had increased bound anti-MBP. Seven of these 111 patients with abnormal free and/or bound anti-MBP had nor-

Table 2. Cerebrospinal Fluid Data in Patients with Anti-PLP Associated Optic Neuritis and with Anti-PLP Associated Multiple Sclerosis

Patient No.	Diagnosis	Intra BBB IgG Synthesis			Anti-MBP		Anti-PLP	
		IgG Index[a]	Daily Synthesis[b]	OB[c]	Free	Bound	Free	MRI (Brain)
2572	Optic neuritis	0.70	1.90	Neg	1	0	11	Abnormal
1071	Optic neuritis	0.62	−2.16	Neg	0	0	10	ND
1086	Optic neuritis	0.50	−4.50	Neg	3	1	13	ND
2423	Optic neuritis	0.61	1.40	Neg	2	4	7	Abnormal
937	Optic neuritis	0.84	1.90	Neg	4	2	12	ND
2800	Optic neuritis	0.76	1.16	Neg	3	2	9	ND
3069	MS relapse							
	1988	0.50	−2.35	Pos	4	1	12	Abnormal
	1989	0.55	−0.76	Pos	2	1	15	ND
	1990	0.59	0.00	Pos	3	7	0	ND
3267	MS progressive	0.54	−2.03	Neg	0	0	15	ND
3307	MS relapse	0.43	−4.22	Neg	0	0	17	Abnormal
3774	MS progressive	0.49	−4.55	Neg	1	0	13	Abnormal
3778	MS relapse	0.64	3.19	Neg	1	1	14	Abnormal
3827	MS relapse	0.49	−3.60	Neg	0	0	15	Questionable
3829	MS relapse	0.61	−2.50	Pos	0	0	14	ND
3825	MS progressive	0.50	−3.50	Neg	0	0	10	ND
3833	MS progressive							
	1991	0.42	−5.27	Pos	0	0	15	ND
	1993	0.51	−1.72	Pos	0	0	17	ND

Cerebrospinal fluid data of 15 individual patients with anti-proteolipid protein (anti-PLP) associated forms of optic neuritis or multiple sclerosis. Anti-myelin basic protein (anti-MBP) and anti-PLP results expressed in radioactivity units; see Methods for definition and range.

[a]Link's IgG index.
[b]Tourtellotte's empirical formula for daily IgG synthesis.
[c]Oligoclonal banding by polyacrylamide gel isoelectric focusing (pH 3–10). Samples with matching bands in serum due to damaged blood–brain barrier (BBB) are read negative.

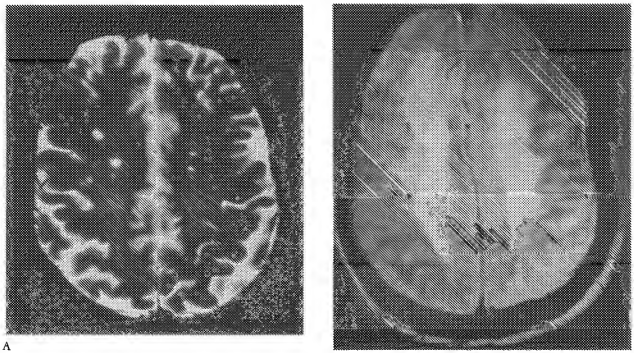
MRI = magnetic resonance imaging; Neg = negative; ND = not determined; Pos = positive.

mal intra BBB IgG synthesis and negative oligoclonal banding. The remaining 5 of 116 patients with progressive MS had undetectable anti-MBP; 4 of these 5 patients had increased anti-PLP, whereas in 1 patient neither antibody was detected. In a group of 89 patients in clinical remission, 47 had an increased IgG index, 49 had increased daily IgG synthesis, and 49 had positive CSF oligoclonal banding. Anti-MBP was slightly elevated in 15 of these 89 patients. None of the patients in clinical remission had increased anti-PLP. Anti-MBP and anti-PLP were not detected simultaneously in any of the 385 MS patients. However, a single patient (Patient 3069, Table 2) with elevated anti-PLP on two occasions (in 1988 and 1989) had, a year later (in 1990), increased anti-MBP but anti-PLP became undetectable.

The uniqueness of detecting increased levels of CSF anti-PLP only in optic neuritis and MS patients with undetectable levels of anti-MBP, stimulated further analysis of this antibody in the context of these diseases.

Optic Neuritis and Multiple Sclerosis Associated with Increased Anti-Proteolipid Protein

CSF data of 6 patients with optic neuritis and 9 patients with MS with increased titers of CSF anti-PLP and undetectable anti-MBP are illustrated in Table 2. Both groups of patients had remarkably normal values of intra BBB IgG synthesis; in the group of 6 optic neuritis patients, with the exception of 3 who had a slightly increased IgG index, daily IgG synthesis and oligoclonal banding of CSF immunoglobulins were normal in all 6. Similarly, all 9 MS patients had normal values for the IgG index and 8 of 9 had normal daily rate of IgG synthesis. Oligoclonal banding of CSF IgG was positive in 3 of the 9 patients (Patients 3069, 3829, and 3833; see Table 2). Furthermore in 1 of these 3 patients (Patient 3069), after repeated sampling, anti-PLP became undetectable whereas anti-MBP titers were elevated (see Table 2). Anti-MBP and anti-PLP were never simultaneously detected in any of the 440 patients with either optic neuritis or MS who participated in this study. MRI of the brain was performed



A B

Fig 2. Magnetic resonance imaging of the brain, of 2 patients with anti-proteolipid protein specific multiple sclerosis (MS). (A) Patient 3774 showing chronic focal MS plaques. (B) Patient 3307 showing diffuse disseminated demyelination of the centrum ovale.

on 7 of the 15 patients with optic neuritis and MS associated with increased CSF anti-PLP; 6 of these 7 patients had abnormal results (see Table 2). These MRI studies showed either focal lesions or more diffuse disseminated disease (Fig 2A, B).

Because these CSF studies suggested two different immune responses associated with optic neuritis and/or MS, the next objective was to obtain CNS tissue from MS patients with either CSF anti-MBP or CSF anti-PLP, to determine if IgG eluted from their brain contained only the homologous antibody detected in CSF, or whether both anti-MBP and anti-PLP were present.

Central Nervous System Tissue Data of Anti-Myelin Basic Protein or Anti-Proteolipid Protein Specific Forms of Multiple Sclerosis

CSF and CNS tissue data of 4 patients who died with MS are illustrated in Table 3. Patient 1490 had increased levels of CSF anti-MBP and undetectable anti-PLP. As expected, his brain, spinal cord, and optic nerve tissues contained large quantities of extracellular, free as well as tissue-bound anti-MBP per milligram of IgG. Furthermore, anti-MBP could be detected in free and tissue-bound whole protein extracts or in purified IgG from these extracts, and it could be further purified by two-step affinity chromatography. Anti-PLP was undetectable in all areas of CNS tissue from this patient. Both Patients 576 and 1312 showed similar CSF and tissue data with elevated IgG index and increased daily IgG synthesis, positive oligoclonal banding of CSF immunoglobulins, as well as increased anti-MBP titers with free/bound ratios above unity and undetectable anti-PLP. Brain tissue from these 2 patients contained high levels of free and tissue-bound anti-MBP and undetectable anti-PLP.

Patient 3307 showed remarkably different results. With the exception of an increased anti-PLP level all other CSF parameters (IgG Index, daily rate of IgG synthesis, and oligoclonal banding) were normal. Brain tissue obtained from this patient contained large quantities of extracellular, free as well as tissue-bound, anti-PLP per milligram of IgG. Anti-MBP was undetectable.

Similar to CSF results, these 4 MS CNS tissue studies confirmed that either anti-MBP or anti-PLP is part of IgG in a given patient at a given time, and simultaneous appearance of both antibodies did not occur. Because anti-MBP was more commonly observed than anti-PLP, the final objective of this report was to determine the epitope range for this autoantibody.

Synthetic Peptide Specificity of Anti-Myelin Basic Protein from Multiple Sclerosis Central Nervous System Tissue

Free and bound anti-MBP was purified by affinity chromatography from spinal fluid and CNS tissue of MS

Table 3. Cerebrospinal Fluid and Central Nervous System Tissue Data of Four Individual Patients Deceased with Multiple Sclerosis

Patient No.	Intra BBB IgG Synthesis		CSF Data				CNS Tissue Data				
				Anti-MBP		Anti-PLP		Anti-MBP		Anti-PLP	
	IgG Index[a]	Daily Synthesis[b]	OB[c]	Free	Bound	Free	Area 1 Preparation	Free	Tissue Bound	Free	Tissue Bound
1490							Brain				
9/85	0.79	23.30	Pos	14	4	0	Whole extract	33	12	0	0
							IgG	26	11	0	0
							Anti-MBP	23	9	0	0
							Spinal cord				
							Whole extract	35	13	0	0
							IgG	25	10	0	0
							Anti-MBP	20	8	0	0
							Optic nerves				
							Whole extract	15	6	0	0
							IgG	10	8	0	0
576											
2/81	2.53	52.12	Pos	11	2	0	Brain				
3/81	2.04	45.72	Pos	13	1	0	Whole extract	34	27	1	0
4/81	2.01	59.96	Pos	12	1	0	IgG	27	25	0	0
5/81	4.08	79.14	Pos	12	1	0	Anti-MBP	25	17	0	0
1312											
2/83	0.79	23.30	Pos	14	4	0	Brain				
3/83	0.89	49.40	Pos	10	5	0	Whole extract	27	20	0	0
5/83	0.89	75.70	Pos	15	3	0	IgG	20	15	0	0
6/83	0.92	59.30	Pos	12	4	0	Anti-MBP	17	10	0	0
3307											
11/85	0.47	−2.80	Neg	0	0	15	Brain				
9/89	0.43	−4.22	Neg	0	0	17	Whole extract	0	0	32	22
							IgG	0	0	30	20

Cerebrospinal fluid (CSF) and central nervous system (CNS) tissue data of 4 individual patients deceased with multiple sclerosis. Patients 1490, 576, and 1312 had anti-myelin basic protein (anti-MBP) in their CSFs and CNS tissues, whereas Patient 3307 had anti-proteolipid protein (anti-PLP) in CSF and CNS tissue. Anti-MBP and anti-PLP in CNS tissue expressed per milligram of IgG.

[a]Link's IgG index.
[b]Tourtelotte's empirical formula for daily IgG synthesis.
[c]Oligoclonal banding by polyacrylamide gel isoelectric focusing (pH 3–10). Samples with matching bands in serum due to damaged blood–brain barrier.

Pos = positive; Neg = negative.

Patient 1490. The purified antibody was reacted with 53 synthetic peptides of human MBP (Fig 3). Some of these peptides produced complete inhibition of antibody (●—●), some produced partial inhibition (⊢—, —●), and some did not react at all with purified anti-MBP (—). Peptides from set 1 corresponding to residues 75 to 106 of hMBP produced complete inhibition of free and bound antibody from CSF or CNS tissue (lines A and B, Fig 3); peptides corresponding to residues 61 to 83 produced incomplete inhibition of free antibody (lines A and B, Fig 3).

To further confirm these observations and to narrow the epitope, free and bound anti-MBP purified from the same CNS tissue were reacted with a set of more refined synthetic peptides (set 2) each of 15 residues and overlapping 7 residues (line C, Fig 3). Similar results were observed. Once again synthetic peptides corresponding to overall residues 84 to 105 completely inhibited free and bound antibody (●—●), whereas peptides corresponding to residues 63 to 91 partially inhibited free anti-MBP (⊢—●, ⊢—⊣).

Because tissue-bound anti-MBP had a greater synthetic peptide specificity, it was then reacted with a third set (set 3) of 11 synthetic peptides (line D, Fig 3). This set of peptides corresponds to residues 75 to 95 situated proximal to the triproline sequence of human MBP (99, -100, -101). As the length of these peptides was reduced serially by a single residue, their anti-MBP binding ability progressively decreased. These results are suggesting that the center of the epitope of anti-MBP purified from MS tissue is located between residues 80 to 100 although the whole epitope may be located anywhere between 61 and 106. Synthetic peptides corresponding to residues located

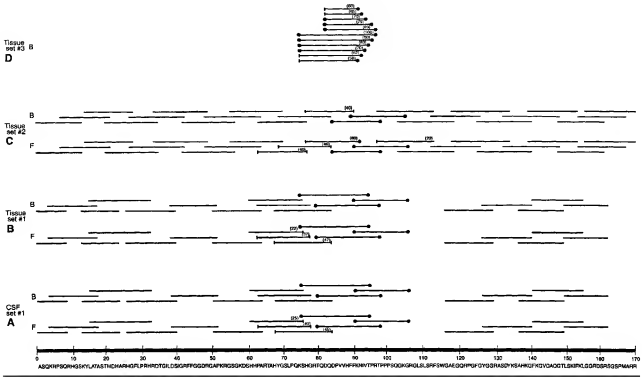


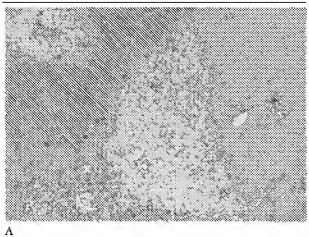
Fig 3. Synthetic peptide specificity of free and bound anti-myelin basic protein (MBP) purified from cerebrospinal fluid (CSF) and central nervous system tissue obtained from a single multiple sclerosis patient. Solid, thick bar (0–170) = human MBP molecule with its 170 amino acid residues (single letter system); short, thin bars = synthetic peptides; numbers in brackets = percentage of inhibition of antibody. (●—●) = complete inhibition (70–100%); (●—) = partial inhibition (45–70%); (—) = 20–45%; (—) = no inhibition (0–20%). Set 1 = 18 synthetic peptides of different sizes (18–25 residues) covering most of the human MBP molecule: set 2 = 24 synthetic peptides of equal sizes (15 residues each) covering the entire length of human MBP molecule and overlapping each other by 7 residues; set 3 = 11 synthetic peptides of different sizes (8–21 residues) covering the area between 75 and 95 of human MBP.

at either end of the molecule (1–60 and 110–170) did not bind anti-MBP.

Discussion

If MS is an autoimmune-mediated disease, the results and observations of this report are supporting the concept of at least two immunologically distinct forms, i.e., a more common form associated with increased CSF and tissue anti-MBP and a less frequent form associated with elevated levels of anti-PLP. Similar to our previous reports, the majority of patients with optic neuritis and/or MS who participated in this study had increased levels of CSF anti-MBP [17, 21] and undetectable anti-PLP. However, a small number of patients with optic neuritis or active MS who had underectable CSF anti-MBP have now been observed to have autoantibodies to PLP. Anti-MBP and anti-PLP were not detected simultaneously in any patient, but they were found in 1 patient (Patient 3069; Table 2) at different moments in time when serial sampling was performed. Further longitudinal studies with repeated CSF analyses to detect autoantibodies to a more complete series of myelin proteins are essential for elucidating the autoimmune process associated with MS demyelination.

The clinical profiles of the 9 patients with anti-PLP associated MS were variable but highly characteristic of MS. These patients may have shown either single attacks of optic neuritis, or attacks of paresthesia and dysesthesia, or progressing spastic ataxic syndrome, or progressing spastic paraplegia to the point of complete leg paralysis as well as general cognitive dysfunction. MRIs of their brain showed abnormalities characteristic of MS (see Fig 2A, B). Of all patients with anti-PLP specific MS, Patient 3307 (see Tables 2 and 3) had the most perplexing psychiatric and medical history: As a religious nun, at age 27, she first experienced transient weakness of her legs for a period of 2 to 3 months. During the last 15 years of her life, her leg paralysis increased until she was confined to a wheelchair and subsequently to a chronic hospital bed. Attacks of manic–depressive psychosis were replaced by psychomotor retardation and she experienced repeated emergency admissions to the hospital for convulsions and coma associated with hyponatremia. MRI of her brain showed diffuse, disseminated demyelination of the



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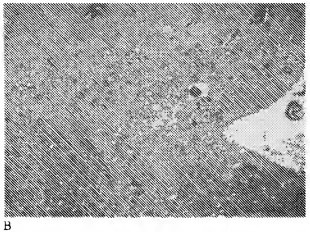
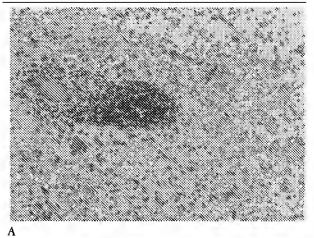


B

Fig 4. Plaques from a patient (Patient 3307) with chronic, progressing multiple sclerosis. These plaques contain anti-proteolipid protein and no anti-myelin basic protein. (A) Small plaques of chronic demyelination in the periventricular white matter of the right temporal horn. The plaques characteristically surround a central vein and are associated with a paucity of lymphocytic cuffing. (Luxol fast blue/hematoxylin and eosin, original magnification × 280.) (B) Margin of a chronic demyelinative plaque (P) *extending from the optic chiasm into the right optic tract* (OT) *and encroaching upon the supraoptic nucleus* (SN). *This plaque may have accounted for hyponatremic attacks. (Luxol fast blue/hematoxylin and eosin, original magnification × 175.)*



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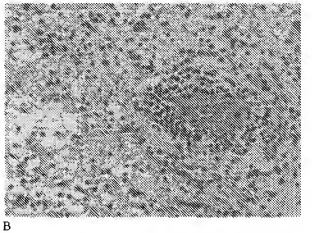

B

Fig 5. Plaques from 2 patients with rapidly progressive forms of multiple sclerosis, containing anti-myelin basic protein and no anti-proteolipid protein. Both plaques show prominent perivenular lymphocytic cuffing, severe demyelination associated with microvacuolation, and astrocytic proliferation. (Luxol fast blue/hematoxylin and eosin, original magnification × 360.) (A) Patient 576. (B) Patient 1312.

centrum ovale (see Fig 2B). She died at age 59 and neuropathological examination showed MS plaques with very little inflammation (Fig 4A, B). Plaques were located in the hypothalamus adjacent to the paraventricular and supraoptic nuclei and this may have accounted for her hyponatremic attacks (Fig 4B). CSF sampled 2 years before her death showed a single abnormality, i.e., increased anti-PLP; cell count, absolute and relative levels of IgG, oligoclonal banding, and anti-MBP titers were all normal.

IgG extracted from CNS tissue of 4 individual, clinically definite, neuropathologically confirmed MS patients, 3 with increased CSF anti-MBP and no anti-PLP, and 1 with CSF anti-PLP and undetectable anti-MBP, contained only the homologous antibody from CSF, further supporting the concept of two immunologically distinct forms of MS.

Neuropathological assessment of these 4 patients confirmed that anti-MBP associated MS contained a greater degree of inflammation of the brain than anti-PLP associated MS (Figs 4 and 5). However, the high degree of inflammation observed in the 3 patients with anti-MBP associated MS may be due to the aggressive forms of these particular cases. Further studies of classical and more benign cases of MS are in progress. Although some cases of anti-MBP associated MS may have relatively less inflammation, we are anticipating that abundant inflammation will not be found in anti-PLP associated MS.

Determination of the immune specificity toward MBP or PLP in individual MS patients is extremely important because it is likely to lead to specific therapies.

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Autoreactive T Lymphocytes in Multiple Sclerosis Determined by Antigen-induced Secretion of Interferon-γ

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Abstract

Multiple sclerosis (MS) is a disease with unknown cause characterized by inflammation and demyelination in the central nervous system. Although an autoimmune pathogenesis has been suggested, there are no conclusive data on the number of T cells autoreactive with myelin antigens in MS compared to controls. We showed that T lymphocytes secreting interferon-γ in response to possible target autoantigens are severalfold more common among PBL mononuclear cells in patients with MS than in patients with aseptic meningitis and tension headache. On average T cells reactive with myelin basic protein (MBP), two different MBP peptides, or with proteolipid protein amounted to 2.7–5.2/10⁵ PBL from MS patients. MBP-reactive T cells were still more frequent among mononuclear cells isolated from the cerebrospinal fluid (CSF; 185/10⁵ CSF cells). We concluded that T cells reactive with myelin autoantigens are strongly increased in MS. This approach to detect them could allow definition of immunodominant T cell epitopes in individual MS patients, and thereby enable further development towards specific immunotherapy. (*J. Clin. Invest.* 1990. 86:981–985.) Key words: autoimmunity • demyelination • cerebrospinal fluid

Introduction

An autoimmune pathogenesis for multiple sclerosis (MS)[1] has been suggested due to clinico-pathological similarities with experimental allergic encephalomyelitis (EAE). Induction of EAE depends on $CD4^+$ T cells that are autoreactive with myelin proteins (1). Myelin basic protein (MBP) (2) and proteolipid protein (PLP) (3, 4) have both been shown to be encephalitogenic. Previous data on T cell reactivities to myelin antigens in MS (5–15) are partly inconsistent. One reason for failures to detect such reactivities (7–9) may be that T cells recognizing myelin antigens constitute a minute proportion of the circulating T cells. Even in EAE, the number of MBP reactive T cells were as low as $3–4/10^4$ lymphocytes when isolated at the target (16). In MS, nonselective T cell cloning with lectins shows no myelin reactivity (7, 8, 10) but is readily demonstrable in culture conditions selecting for T cell reactivity against MBP (6, 8, 11–13, 15). Since MBP reactive T cells also have been cloned from healthy individuals (5, 12) the relevance of these observations is unclear. However, selected T cell cloning does not generally allow quantitation of T cell responses, which may be necessary to evaluate their importance. We were able to circumvent these problems by taking advantage of the fact that T cells may secrete IFN-γ in response to the presented antigen (17). By applying an immunospot assay where such cells can be counted (18, 19) it was possible to estimate the number of T cells reactive with autoantigens, in this case different myelin antigens, both in MS patients and controls. Furthermore, this particular cytokine is of potential importance as effector molecule in MS. A therapeutic trial in MS with systemic administration of IFN-γ led to exacerbations and general immune activation (20).

Since the immune response in neuroinflammatory diseases have been shown to be compartmentalized to the target for immune attack (21–24), we examined mononuclear cells from the cerebrospinal fluid (CSF) in addition to PBL.

Methods

Patients. Specimens of CSF and peripheral blood were obtained from 39 untreated patients (29 females) with clinically definite MS. Their ages were 17–68 yr (mean 42). In 33 of them CSF was examined. It was considered relevant to use samples from controls with and without neuroinflammatory disease. 16 patients (nine females) had acute aseptic meningitis (AM). Their ages were 18–80 yr (mean 43). Samples were taken between 1–8 wk after clinical onset. CSF was examined in 15 of the AM patients. 25 patients (20 females) had tension headache (TH). Their ages were 29–69 (mean 47). They lacked physical or laboratory signs of organic disease. CSF was accessible for examination in seven of these.

Assay for single cells secreting IFN-γ in response to antigen. The principles for immunospot enumeration of individual secretory cells using 96-well nitrocellulose bottomed microtiter plates (Millititer-HAM, Millipore Continental Water Systems, Bedford, MA) were followed (18, 19). Wells were coated with 100-μl aliquots of mouse monoclonal anti-human IFN-γ antibody (7-B6-1) (25) at 6 μg/ml at 4°C overnight, and washed with PBS, pH 7.4. PBL were prepared on Ficoll Lymphoprep (Nyegaard, Oslo, Norway) and washed three times in

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1. *Abbreviations used in this paper:* AM, aseptic meningitis; CSF, cerebrospinal fluid; EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; PLP, proteolipid protein; PPD, purified protein derivate; TH, tension headache.

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tissue culture medium consisting of Iscove's modification of Dulbecco's medium with 2 mM L-glutamine (both from Flow Laboratories, Irvine, U.K.), and 20% (vol/vol) fetal calf serum (Gibco Laboratories, Paisley, U.K.). Mononuclear cells from 20 ml of CSF, sampled into siliconized glass tubes, were pelleted by centrifugation for 10 min at 200 *g*. The cells were resuspended and washed in tissue culture medium. 200-μl aliquots of PBL (10^6/ml, 2×10^5 cells) or CSF cells ($5–20 \times 10^5$ cells) were added to appropriate wells of the plates. Antigens or lectins were added in 10-μl aliquots to a final concentration of 10 μg/ml. This concentration of antigen gave a maximum number of spots in preliminary experiments.

To control wells for each patient the antigen was omitted. MBP (26) and PLP (27) were prepared from bovine brain. PLP was free from any MBP contamination as checked by Western blot (28) and employing a rat polyclonal antiserum against MBP. We had access to two different synthetic peptides of MBP. We arbitrarily named the peptides P_4 and P_6, and their respective amino acid sequences corresponded to 132–150 and 174–191 of mouse MBP (29). They are homologous to the human protein except for a single substitution at position 146 (Lys to Arg). These peptides were synthesized on a peptide synthetizer (model 430A; Applied Biosystems, Inc., Foster City, CA), followed by purification on HPLC employing reverse phase chromatography on a C-18 column eluted with acetonitrile gradient (0–60%) in 0.1% trifloruoacetic acid. Amino acid analysis confirmed the correct product. Purified protein derivate (PPD) (Statens Serum Institute, Copenhagen, Denmark) and PHA (Difco Laboratories, Inc., Detroit, MI) were used as positive control antigen and mitogen, respectively. Because of limited access to CSF cells, only cultures with added MBP and without any added antigen could be analyzed for cells from this compartment. After a 48-h culture at 37°C, 7% CO_2, and humid atmosphere the plates were washed with PBS. 100-μl aliquots of a rabbit polyclonal anti-human IFN-diluted 1/500 (Interferon Sciences, New Brunswick, NJ) were added. After washing, biotinylated anti-rabbit IgG-diluted 1/1000 (Vector Laboratories, Inc., Burlingame, CA) was added followed by an avidin-biotin peroxidase complex diluted 1/200 (ABC Vectastain-Elite Kit; Vector Laboratories, Inc.) for 1 h. After peroxidase staining (24), spots which corresponded to cells that had secreted IFN-γ were enumerated with a dissection microscope. No spots appeared in specificity control experiments in which the capture antibody was changed to an irrelevant mouse monoclonal antibody or the rabbit polyclonal antibody omitted. To calculate the number of T cells responding to a particular antigen, numbers of spots in cultures with no antigen added were subtracted from the numbers of spots in the different cultures exposed to antigen.

The data are expressed as numbers of spots/10^5 mononuclear cells. Mann-Whitney's U-test was used for statistical evaluation.

Results

After a 48-h culture of PBL or CSF mononuclear cells and immuno-enzyme staining for secreted IFN-γ, red-brown spots appeared that were easy to count in a dissection microscope. The number of memory T cells responding specifically to an antigen were estimated after subtraction of the values obtained in cultures without antigen.

T cells reactive with the different myelin autoantigens were present in peripheral blood of MS patients (Table I; Fig. 1). On average, 2.7 cells among 10^5 peripheral blood mononuclear cells responded to MBP. Such cells were also found in blood from approximately one third of the two control groups, but at much lower numbers. On average the number of MBP reactive cells in blood were about sevenfold more common in MS than in AM or TH. An ~ 10-fold higher number of T cells reactive with PLP in MS as compared with the control groups were found in blood specimens (Table I). The numbers of T cells responding to PPD and PHA did not differ significantly between the groups of patients.

To study whether T cell reactivity against certain peptides was detectable with the present methodology, and if there are any indications of occurrence of immunodominant epitopes in MS, we used two synthetic peptides, P_4 (amino acid residues 132–150 according to mouse sequence, part of MBP exons 5 and 6, and encephalitogenic in guinea pig) (29), and P_6 (amino acid residues 174–191, part of MBP exons 6 and 7, and no described encephalitogenicity) (29). As shown in Table I, the group of MS patients had ~ 10-fold higher numbers of T cells recognizing these peptides as compared with the two control groups. In Fig. 1, antigen reactivities in individual MS patients are plotted. Most patients showed similar reactivity to the two different peptides, however, a few patients showed a preferential but not exclusive reactivity to one of the peptides. There was generally a higher response to the peptides than to native MBP.

Due to the limited numbers of CSF cells available for analysis, only MBP reactive specific memory T cells could be examined (Table I; Fig. 2). Again, the number of spots obtained from culture wells with added MBP exceeding those with no added antigen were calculated from samples of individual patients. In this way, the number of MBP reactive T cells were on

Table I. Numbers of Antigen or Lectin-induced Interferon-γ Secreting Cells/10^5 Mononuclear Cells

		Cerebrospinal fluid	Peripheral blood					
Patient group		MBP	MBP	MBP-P_4	MBP-P_6	PLP	PHA	PPD
Multiple sclerosis	mean±SD	185±320	2.7±2.7	5.2±9.5	4.9±9.4	4.7±9.4	162.2±148.7	20.1±27.2
	n	33	39	39	37	34	39	39
	P value	0.001/0.001	0.001/0.001	0.001/0.001	0.001/0.001	0.001/0.001	NS	NS
Acute aseptic meningitis	mean±SD	7.6±17	0.4±0.7	0.5±0.9	0.7±1.1	0.5±0.8	122.9±170.8	12.3±17.8
	n	15	16	16	16	13	16	16
	P value	NS	NS	NS	NS	NS	NS	NS
Tension headache	mean±SD	4.3±9.3	0.3±0.6	0.4±0.7	0.5±0.8	0.3±0.8	124.7±157.9	39.9±89.5
	n	7	25	25	25	20	25	

For samples of each individual patient numbers of spots from cultures with no antigen added were calculated and subtracted from the values of the different cultures exposed to antigen. The data are expressed as numbers of spots/10^5 mononuclear cells. Mean values (*m*), standard deviations (*SD*), and numbers of patients (*n*) are shown. The *P* values refer to comparisons between MS with AM/MS with TH patients.

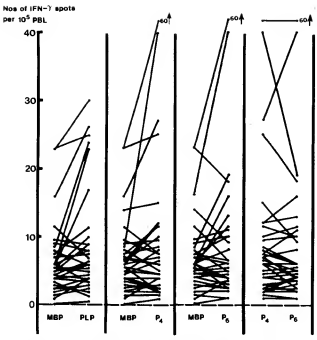

Figure 1. Numbers of spots corresponding to cells/10^5 PBL that have secreted IFN-γ in response to MBP, PLP, MBP-P_4 (P_4), and MBP-P_5 (P_5). The lines connect numbers of spots obtained for each antigen in individual MS patients.

average 185/10^5 mononuclear CSF cells and thus ~ 30-fold more common among MS CSF cells than among CSF cells of patients with AM or TH. Comparing frequencies of MBP reactive T cells in MS blood and CSF, the frequency was ~ 70-fold higher in CSF.

IFN-γ spots resulting from cell cultures with no antigen added (Table II) may represent T cells already activated to cytokine secretion in vivo, or memory T cells responding in vitro to antigen carried with putative antigen presenting cells such as macrophages and B cells also contained in the sample. The patients with AM showed higher numbers of IFN-γ secreting cells in blood, whereas MS patients did not differ in this respect from headache patients. In CSF, however, MS patients had a marked increase in numbers of these cells, while patients with AM did not differ from those with headache (Table II). In view of an ~ 60-fold higher CSF mononuclear cell count in AM compared with headache patients, it can be concluded that AM patients have a prominent increase in IFN-γ secreting cells in CSF. It is also interesting how patients with headache devoid of organic CNS disease signs, showed some cells that produced IFN-γ (Table II).

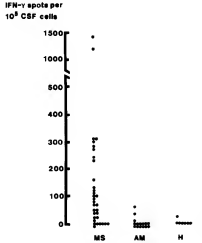

Figure 2. Diagram showing numbers of IFN-γ spots/10^5 CSF mononuclear cells from patients with MS, AM, and TH (*H*) after a 48-h culture in the presence of MBP. The data refer to numbers of values after subtraction from cultures with no antigen added.

Table II. CSF Cell Counts and Numbers of IFN-γ Secreting Cells/10^5 Mononuclear Cells with No Antigen Added

Patient group		Mononuclear cells in CSF	IFN-γ secreting cells: Cerebrospinal fluid	IFN-γ secreting cells: Peripheral blood
		×10^6/liter		
Multiple sclerosis	m̄±SD	6.4±5.7	384±656	4.0±3.9
	n	39	33	39
	P value	ND	0.001/0.001	NS
Acute aseptic meningitis	m̄±SD	87±177	99±178	7.5±4.4
	n	16	15	16
	P value	ND	NS	0.005
Tension headache	m̄±SD	1.5±0.9	97±107	3.0±1.7
	n	7	7	25

Data refer to numbers of spots corresponding to IFN-γ secreting cells/10^5 mononuclear cells, after 48 h culture of cells with no added antigen or lectin.

Discussion

This study shows that the number of circulating T cells reactive with MBP is increased in MS patients as compared with patients with AM and TH. The other important putative autoantigen in MS is PLP due to its experimental encephalitogenicity (3, 4). No significant T cell reactivity in MS with this antigen has previously been found (9, 10). We now show an ~ 10-fold higher number of T cells reactive with PLP in MS as compared with the control groups. These differences were not due to a generally increased capacity to produce IFN-γ in response to antigen or lectin in MS, since the numbers of T cells responding to PPD and PHA did not differ significantly between the groups of patients. The PPD response is compatible with a T cell memory towards BCG, employed as vaccine in Sweden in the age groups examined. The PHA response represents a polyclonal T cell activation that is known to promote IFN-γ production (19).

During antigen processing the native protein is cleaved into short peptides that $CD4^+$ cells recognize when presented on MHC class II molecules (30). The $CD4^+$ T cells appear to preferentially respond to peptides that bind with high affinity to the MHC class II (31). This may be the reason for the differences observed in encephalitogenicity of various MBP peptides in different animal strains. For example, the encephalitogenic peptide in Lewis rats comprises amino acid residues 68–88 of MBP (32), strain 13 guinea pigs residues 114–122 (33), and SJL/J mice residues 87–101 (34) and PL/J, residues 1–11 (35). In the genetically heterogenous population of MS patients, such immunodominant epitopes (if present) may be

expected to differ between individuals. If such immunodominant and putatively pathogenetic peptides could be defined for each individual MS patient, specific immunotherapy could be tried as has been done successfully in EAE (36–39). With the two arbitrarily chosen peptides used here, a few patients showed a preferential but not exclusive reactivity to one of the peptides. There was a generally higher response to the peptides than to native MBP. The reason for this is unclear. Thus, individual MS patients respond to different nervous tissue proteins, and even to different peptides within the same protein. This indicates the presence of different MBP reactive T cell clones. On the other hand, only a minor fraction of possible peptides that can be immunodominant has been examined. We currently employ the present methodology to examine T cell reactivities towards a multitude of different peptides of MBP and PLP.

In organ specific experimental autoimmune diseases such as EAE, there are indications of an enrichment of the target directed immune response to the afflicted organ (23). In MS, we recently showed elevated numbers of plasma cells producing autoantibodies against myelin antigens in CSF, but not in blood (24). Such a sequestration of the immune response to target may also occur for MBP-reactive T cells in MS since such cells were ~ 70-fold more common among CSF cells than PBL. This could either be due to selective recruitment of MBP-reactive T cells to the CSF compartment or to a local clonal expansion.

Autoreactive T cells in MS may represent a primary cause of disease or may be the consequence of inflammatory CNS damage. The latter possibility may be valid at least partly since we have observed autoreactive T cells in acute cerebrovascular diseases using the same antigens and methodology (data not shown). However, this finding does not rule out a pathogenetic role of autoreactive T cells in MS. In genetically predisposed individuals, persistence of such cells at high numbers may contribute to immune-mediated tissue damage.

IFN-γ has a special interest as an effector molecule in MS. It has previously been unsettled whether its production in MS is defective, unchanged, or increased (40). IFN-γ is a potent immunoregulatory cytokine. Major effects include enhancement and induction of class I and II MHC gene expression (41), activation of macrophages (42, 43), and induction of molecules involved in T cell homing (44). Cytokines such as IFN-γ usually act locally, and their production in vivo is poorly reflected in body fluids such as serum or CSF. As in this study, these problems can partly be circumvented by isolating inflammatory cells and monitoring their IFN-γ production in vitro after short-term culture. If our data with cell cultures with no antigen added represent T cells activated to IFN-γ secretion in vivo, patients with MS have a marked increase in intrathecal IFN production. The antigen specificity of these in vivo activated T cells remains unknown. The occurrence of some CSF cells that produced IFN-γ also in patients with headache lacking organic CNS disease, is compatible with the hypothesis that systemically activated T cells normally pass the blood-brain barrier possibly to ensure immune surveillance of the brain (45).

In conclusion, T cells autoreactive with different myelin components are elevated in peripheral blood and strongly enriched in CSF of patients with MS. A future use of a broad spectrum of peptide antigens to detect autoreactive T cells may allow definition of immunodominant epitopes that are candidates for specific immunotherapy in individual patients. We also expect that the single cell assay may be useful for definition of target antigens in other human diseases in which autoimmune mechanisms are suspected to operate.

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